



## **Controlled enzyme catalyzed heteropolysaccharide degradation**

Xylans

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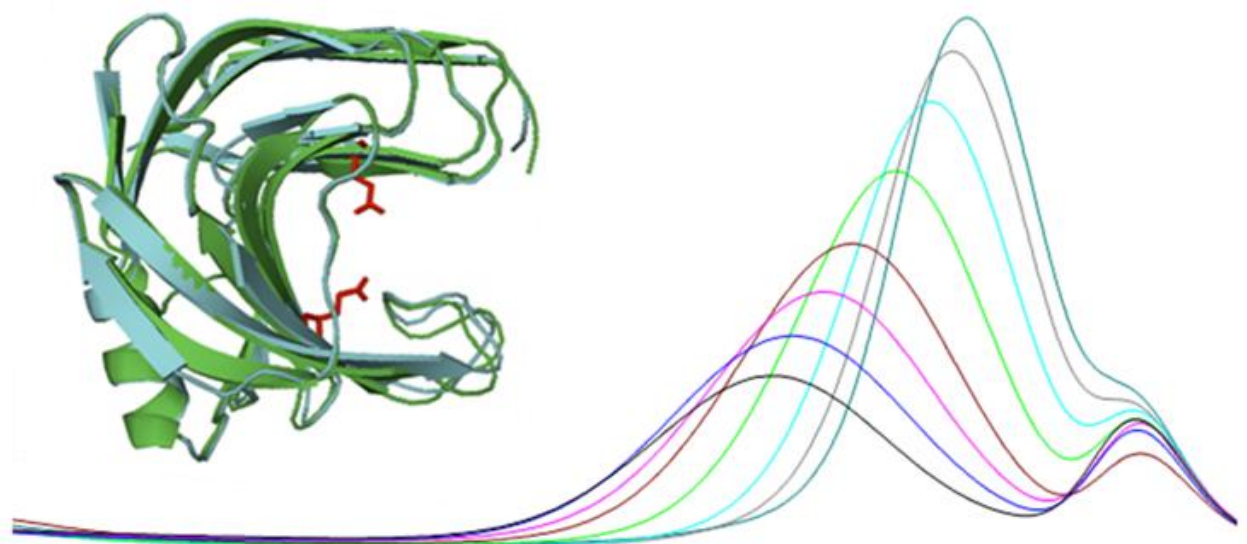
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# Controlled enzyme catalyzed heteropolysaccharide degradation: Xylans



Ph.D. Thesis

Louise Enggaard Rasmussen

March 2011

## **Preface**

This Ph.D. was carried out at BioProcess Engineering, Department of Chemical and Biochemical Engineering, DTU in the period March 2007 – March 2011.

This study was partly supported by the Innovative Bioprocess Technology Research Consortium financed by the Danish Research Council for Technology and Production Sciences, Chr. Hansen A/S, Danisco A/S, Novozymes A/S. Financial support from the FOOD Denmark Graduate School, Center for Advanced Food Studies, Denmark, via DTU, is also acknowledged.

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I sincerely thank all the staff at BioProcess Engineering for creating a supportive working atmosphere and for the nice moments we have shared.

Finally, my loving thanks belong to my husband, Janus, and our sons, Tobias, Johan and Kasper.

The thesis is submitted in fulfillment with the requirements of the degree of PhD in Enzyme Technology.

Louise E. Rasmussen

14<sup>th</sup> of March, 2011

## Abstract

The work presented in this PhD thesis has provided a better understanding of the enzyme kinetics and quantitative phenomena of the hydrolysis of xylan substrates by selected pure enzyme preparations. Furthermore, the options for producing specific substituted xylo-oligosaccharides from selected substrates by specific xylanase treatment have been examined.

The kinetics of the enzymatic degradation of water-extractable wheat arabinoxylan (WE-AX) during designed treatments with selected monocomponent enzymes was investigated by monitoring the release of xylose and arabinose. The results of different combinations of  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55), one derived from *Aspergillus niger* (AF<sub>An</sub>) and one from *Bifidobacterium adolescentis* (AF<sub>Ba</sub>), a  $\beta$ -xylosidase (EC 3.2.1.37) from *Trichoderma reesei*, and a D11F/R122D variant of an endo-1,4- $\beta$ -xylanase (EC 3.2.1.8) from *Bacillus subtilis* (BsX<sub>mut</sub>) were examined. The selected arabinofuranosidases catalyze liberation of arabinofuranosyl residues linked 1 $\rightarrow$ 3 to singly (AF<sub>An</sub>) or doubly (AF<sub>Ba</sub>) substituted xylopyranosyl in arabinoxylan, respectively.

AF<sub>Ba</sub> catalyzed the release of more arabinose, i.e. had a higher rate constant than AF<sub>An</sub>, when added to arabinoxylan at equimolar levels. With respect to the xylose release, AF<sub>An</sub> exhibited a better synergistic effect than AF<sub>Ba</sub> with  $\beta$ -xylosidase. The differences in the synergistic effect could be related to the different mode of action for the two arabinofuranosidases: AF<sub>An</sub> enhanced the probability of more unsubstituted xyloses at (or near) the non-reducing ends for  $\beta$ -xylosidase to attack. AF<sub>Ba</sub> catalyzed the removal of 1 $\rightarrow$ 3 linked arabinofuranosyl, but the  $\beta$ -xylosidase still could not work on the xylan backbone, because there was a  $\alpha$ -1 $\rightarrow$ 2 linked arabinofuranosyl blocking the binding site. However, the synergistic effects between  $\beta$ -xylosidase and the  $\alpha$ -L-arabinofuranosidases on the xylose release were low as compared to the effect of xylanase addition with  $\beta$ -xylosidase, which increased the xylose release by ~25 times in 30 minutes. At equimolar addition levels of the four enzymes, the xylanase activity was thus rate-limiting for the  $\beta$ -xylosidase catalyzed depolymerization to release xylose from arabinoxylan. Thus, the provision of more (unsubstituted) non-reducing ends resulting from xylanase action was more efficient to boost the  $\beta$ -xylosidase activity than provision of more (randomly) unsubstituted xyloses in the arabinoxylan backbone.

The kinetics and substrate selectivity of the *B. subtilis* wildtype xylanase, BsX, which is sensitive to inhibition by TAXI, and the engineered variant, BsX<sub>mut</sub>, which is much less inhibited by TAXI, was examined in order to elucidate the influence of the structural point

mutations. Three dimensional structures of both xylanases were superimposed to elucidate the structural basis for differences in their hydrolytic properties. The comparison showed that the D11F mutation appeared to cause a slight narrowing of the entrance to the active site cleft because the phenylalanine was more bulky than the aspartic acid. The two xylanases were incubated individually with WEAX, water-unextractable arabinoxylan (WUAX), birchwood xylan, and wheat bran, respectively. At equimolar addition, the activity of BsX<sub>mut</sub> was lower than that of BsX with respect to both the initial rate and the product yields obtained after prolonged reaction on the xylan substrates. The lower activity could be related to steric hindrance caused by the D11F mutation. The calculated substrate selectivity factors indicated that BsX and BsX<sub>mut</sub> both had higher catalytic rate on WUAX than on WEAX. Addition of a 100:1 (TAXI:xylanase) molar ratio of the inhibitor confirmed the significantly decreased inhibition of BsX<sub>mut</sub> by TAXI. Addition of TAXI also influenced the xylanases' selectivity factor differently.

In order to assess the heterogenous structure of the substrate matrix and the change occurring during the xylanolytic reaction, the possibilities for using high-performance size exclusion chromatography (HPSEC) as a quantitative method to assess xylo-oligosaccharide profiles was examined. HPSEC is a widely used method for the *qualitative* profiling of oligosaccharide mixtures. A novel method employing HPSEC for the *quantitative* analytical profiling of the progress of enzymatic hydrolysis of different xylan substrates was developed. The method relies on dividing the HPSEC elution profiles into fixed time intervals and utilizing the linear refractive index response (area under the curve) of defined standard compounds. In order to obtain optimal high-performance size exclusion chromatography profiles, the method was designed using 0.1 M CH<sub>3</sub>COONa in both the mobile phase and as the sample solution. This was based on the systematic evaluation of the influence of the mobile phase, including the type, ionic strength and pH, on the refractive index detector response. A time study of the enzyme catalyzed hydrolysis of birchwood xylan and wheat bran by BsX was used as an example to demonstrate the workability of the new HPSEC method for obtaining progress curves describing the evolution in the product profile during enzyme catalysis.

Flaxseed mucilage (FM) has recently been reported to contain an interesting structure, notable a mixture of highly doubly substituted arabinoxylan as well as rhamnogalacturonan I (RGI) with unusual side group substitutions. This substrate was therefore evaluated as a potential substrate for the production of xylo-oligosaccharides catalyzed by BsX. Treatment of FM with BsX resulted in limited depolymerization, but when BsX and FM were incubated together on WE-AX, WUAX and birchwood xylan, significant amounts of xylose were released. Moreover, arabinose was released from both WE-AX and WU-AX. Since no xylose

or arabinose was released by BsX addition alone on these substrates, nor without FM or BsX addition, the results indicate the presence of endogenous  $\beta$ -D-xylosidase and  $\alpha$ -L-arabinofuranosidase activities in FM. FM also exhibited activity on both *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside (pNPA) and *p*-nitrophenyl  $\beta$ -D-xylopyranoside (pNPX).

The potential of producing glucurono-xylo-oligosaccharides (GXOS) from wheat bran via specific treatment with BsX<sub>mut</sub> was investigated. After the enzyme catalyzed hydrolysis by BsX<sub>mut</sub>, the GXOS were isolated by anion exchange chromatography and the fractions obtained were analyzed for the presence of uronic acid, and by High Performance Anion Exchange Chromatography (HPAEC) and LC/MS for structural verification. Since phosphate also co-eluted during the anion exchange chromatography, the amount of phosphate in the fractions was also determined. LC/MS analysis showed that GXOS was isolated from wheat bran but an even larger amount of RGI was present in the obtained samples together with phosphate. Therefore, further purification has to be made in order to obtain GXOS.

## Dansk sammenfatning

Denne Ph.D. afhandling har undersøgt enzym-kinetikken og hydrolysen af forskellige xylan substrater vha. udvalgte monokomponente enzymer. Via specifik xylanase behandling er muligheden for at producere xylo-oligosaccharider fra udvalgte substrater blevet undersøgt.

Kinetikken for den enzymatiske nedbrydning af opløselig hvede arabinoxylan (WE-AX) med udvalgte monokomponent enzymer blevet undersøgt ved at måle frigivelsen af xylose og arabinose. Forskellige kombinationer af to  $\alpha$ -L-arabinofuranosidaser (EC 3.2.1.55) fra hhv. *Aspergillus niger* (AF<sub>An</sub>) og *Bifidobacterium adolescentis* (AF<sub>Ba</sub>) samt en  $\beta$ -xylosidase (EF 3.2.1.37) fra *Trichoderma reesei*, og en D11F/R122D variant af en endo-1,4- $\beta$ -xylanase (EC 3.2.1.8) fra *Bacillus subtilis* (BsX<sub>mut</sub>) blev undersøgt. Arabinofuranosidaserne katalysere frigivelsen arabinose, der er bundet 1 $\rightarrow$ 3 til henholdsvis enkelt (AF<sub>An</sub>) eller dobbelt (AF<sub>Ba</sub>) substitueret xylose i arabinoxylan.

AF<sub>Ba</sub> katalyserede frigivelsen af mere arabinose, dvs. havde en højere hastigheds konstant end AF<sub>An</sub>, når de var tilsat i ækvimolære mængder til WE-AX. Med hensyn til xylose frigivelsen, udviste AF<sub>An</sub> en bedre synergy end AF<sub>Ba</sub> med  $\beta$ -xylosidase. Forskellen i synergien kan være relateret til de forskellige hydrolysemåder for de to arabinofuranosidaser: AF<sub>An</sub> øger sandsynlighed for flere usubstituerede xylose enheder ved (eller tæt på) de ikke-reducerende ender som  $\beta$ -xylosidase kan hydrolysere.  $\beta$ -Xylosidasen kunne ikke hydrolysere xylankæden efter tilsætning af AF<sub>Ba</sub>, fordi der stadig var  $\alpha$ -1 $\rightarrow$ 2 bundet arabinose som blokerede bindingsstedet. I relation til xylose frigivelsen, så var synergien mellem  $\beta$ -xylosidasen og  $\alpha$ -L-arabinofuranosidaserne dog relativ lav i forhold til synergien mellem xylanasen og  $\beta$ -xylosidasen, hvor frigivelsen af xylose blev øget ca. 25 gange på de første 30 minutter. Ved ækvimolær dosering af de fire enzymer, var xylanase aktivitet således hastighedsbegrænsende for den  $\beta$ -xylosidase katalyseret frigivelse af xylose fra WE-AX. Stigningen i usubstituerede ikke-reducerende ender som følge af xylanase aktiviteten var således mere effektiv til at øge  $\beta$ -xylosidase aktiviteten end stigningen af flere usubstituerede xylose enheder i selve WE-AX polymeren katalyseret af AF<sub>An</sub>.

Kinetikken og substrat selektiviteten for vildtype xylanasen fra *B. subtilis*, BsX, der er sensitiv for TAXI-inhibering og varianten BsX<sub>mut</sub>, som er langt mindre inhiberet af TAXI, blev undersøgt med henblik på at belyse indflydelsen af de strukturelle punktmutationer. 3D strukturer af begge xylanaser blev sammenlignet for at belyse det strukturelle grundlag for forskelle i deres hydrolytiske egenskaber. Sammenligningen viste, at D11F mutationen tilsyneladende forårsagede en mindre indsnævring af indgangen til "active site" kløften, fordi phenylalanin rumligt fylder mere end asparagin syre. De to xylanases blev inkuberet med

henholdsvis WEAX, uopløselig arabinoxylan (WUAX), birke-xylan og hvedeklid. Ved ækvimolær dosering havde BsX<sub>mut</sub> en lavere aktivitet end BsX på alle xylan sybstrater både med hensyn til den initiale hastighed og udbytte. Den lavere aktivitet er sandsynligvis relateret til den steriske hindring, som D11F mutationen udgør. De beregnede substrat selektivitet faktorer for BsX og BsX<sub>mut</sub> angav en højere katalytisk aktivitet på WU-AX end på WE-AX for begge enzymer. Tilsætning af 100:1 (TAXI: xylanase) molforhold af inhibitoren bekræftede BsX<sub>mut</sub>'s nedsatte sensitivitet for TAXI. Tilsætning af TAXI påvirkede ligeledes xylanase selektivitet faktoren forskelligt.

For at kunne undersøge den heterogene struktur af substratet og de ændringer der sker under den xylanase katalyserede reaktionen, blev High-Performance Size Exclusion Chromatography (HPSEC) som en kvantitativ metode undersøgt for at kunne vurdere xylo-oligosaccharid profiler. HPSEC er en udbredt metode til kvalitativ profilering af forskellige polymere. En ny metode blev udviklet, hvor HPSEC bruges til en kvantitativ analyse af forløbet i den enzym katalyserede hydrolyse af forskellige xylan substrater. Metoden bygger på at inddele HPSEC elueringsprofil i bestemte tidsintervaller og udnytte det lineære respons i refraktivt indeks (RI) (areal under kurven) af bestemte standarder. For at have brugbare HPSEC profiler blev 0,1 M CH<sub>3</sub>COONa brugt i både den mobile fase og prøve. Valget af denne mobile fase var baseret på en systematisk undersøgelse af indflydelsen fra den mobile fase, herunder type, ionstyrken og pH på HPSEC kromatogrammet. Et tidsstudie af den BsX katalyserede hydrolyse af birkexylan og hvedeklid blev brugt som et eksempel på anvendeligheden af den nye HPSEC metode til at kunne beskrive udviklingen i substratet og productet.

Hørfrø planteslim (FM) indeholder strukturer, hvor der er relativt meget dobbelt substitueret arabinoxylan samt rhamnogalacturonan I (RGI) med specielle sidegruppe-substitutioner. Dette substrat blev derfor vurderet som et potentiel substrat til den enzyme katalyserede produktion af xylo-oligosaccharider. Tilsætningen af BsX til FM resulterede dog i begrænset depolymerisering, men når BsX og FM blev inkuberet sammen med WE-AX, WUAX og birke-xylan, blev betydelige mængder xylose frigivet. Desuden blev der også frigivet arabinose fra både WE-AX og WU-AX. Xylose eller arabinose blev ikke frigivet af BsX alene på disse substrater, eller fra substraterne alene. Resultaterne påviser således tilstedeværelsen af endogen  $\beta$ -D-xylosidase og  $\alpha$ -L-arabinofuranosidase aktiviteter i FM. Disse aktiviteter var også aktive både på p-nitrophenyl  $\alpha$ -L-arabinofuranoside (pNPA) og p-nitrophenyl  $\beta$ -D-xylopyranoside (pNPX).

Potentialet for at producere glucurono-xylo-oligosaccharider (GXOS) fra hvedeklid vha. BsX<sub>mut</sub> blev også undersøgt. Efter den enzyme katalyseret hydrolyse blev GXOS isoleret ved anionbytter kromatografi og efterfølgende blev fraktionerne analyseret for



tilstedeværelsen af uron syre, High Performance Anion Exchange chromatografi og LC/MS. Da fosfat også co-eluerede under anionbytter kromatografien, blev mængden af fosfat i fraktionerne også bestemt. LC/MS-analyser viste, at GXOS var blevet isoleret fra hvedeklid, men en endnu større mængde af RGI var til stede i fraktionerne sammen med fosfat. Derfor skal der foretages en yderligere rensning af GXOS.

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## List of publications

This thesis is based on the following manuscripts:

### Paper 1:

Endo-1,4- $\beta$ -xylanase activity is rate-limiting for enzymatic xylose liberation from soluble wheat arabinoxylan

Louise E. Rasmussen, Cheng Xu, Jens F. Sørensen, Michael K. Nielsen, Anne S. Meyer

Enzyme and Microbial Technology, submitted.

### Paper 2:

Kinetics and substrate selectivity of a *Triticum aestivum* xylanase inhibitor (TAXI) resistant D11F variant of *Bacillus subtilis* XynA xylanase

Rasmussen L.E., Sørensen J.F., Meyer A.S.

Journal of Biotechnology, 146: 207-214, 2010

### Paper 3:

Size exclusion chromatography for the quantitative profiling of the enzyme-catalyzed hydrolysis of xylo-oligosaccharides

Rasmussen L.E., Meyer A.S.

Journal of Agricultural and Food Chemistry, 58: 762-769, 2010

### Paper 4:

Endogeneous  $\beta$ -D-xylosidase and  $\alpha$ -L-arabinofuranosidase activity in flax seed mucilage

Louise E. Rasmussen, Anne S. Meyer

Biotechnology Letters, 32: 1883-1891, 2010

# Chapter 1 Background

## 1.1 Introduction

Enzymatic hydrolysis of xylans is of considerable importance in several cereal processes ranging from food and biotechnological applications to exploitation of xylans as a carbohydrate source for the fermentation of xylose to biofuels or as a base for development of novel prebiotic food ingredients (Bedford, 2003; Courtin et al., 2008; Pastell et al., 2009; Grootaert et al. 2007; Sørensen et al., 2006; 2007; Van Der Borght et al., 2005). Xylans are the main heteropolysaccharides found in the hemicellulosic part of plant cell walls. They account for 25-35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots, and occur in up to 50% in some tissues of cereal grains (Moure et al., 2006). The degradation of xylan is mainly catalyzed by endo-1,4- $\beta$ -xylanases (xylanases, EC 3.2.1.8) which catalyze the hydrolysis of the  $\beta$ -1,4 linkages in the xylan backbone, generating a mixture of xylo-oligosaccharides (Biely, 1985). The hydrolysis of xylan containing substrates catalyzed mainly by xylanases can be found in a variety of industrial processes.

### 1.1.1 Industrial processes

Xylanases are widely used in the bakery industry, where their main effect is to solubilize the arabinoxylan fraction of the dough. This leads to changes in dough rheology such as development time, consistency, extensibility, and resistance to breakdown. These changes can be seen in the bread as major improvement of the loaf volume and crumb structure (Courtin et al., 2001). Xylanase overdosage leads to sticky and slack doughs as a result of the loss of water holding capacity (Grootaert et al., 2005; Rouau et al., 1994; Trogh et al., 2004, 2005).

In feed for monogastrics, arabinoxylan is believed to result in anti-nutritive effects. The digestive system of these animals lacks the appropriate digestive enzymes which causes an inability to degrade arabinoxylan. These undigested fibers increase the viscosity of the feed in the gut, which interferes with penetration of digestive enzymes, absorption of the digested food and may support pathogenic conditions, especially in broiler chicks (Newkirk et al., 1993). This succession of events leads to a reduction in the digestion rate of cereal based feeds, and subsequently results in an inefficient use of feed and poor growth of the animal. The use of xylanases together with other hemicellulases in animal feed decreases the anti-nutritive effect imposed by the arabinoxylan and also increases the nutritive value of the feed (Courtin et al., 2008).

Xylanases are also important and common processing aids in the industrial wheat gluten-starch separation processes, where the use of xylanases results in higher gluten and starch

yields and improved gluten quality (Christophersen et al., 1997; Weegels et al., 1992). The improved gluten yield is mediated by a decrease in viscosity, which can be attributed to the partial xylanase catalyzed hydrolysis of arabinoxylan (Frederix et al., 2004).

In brewing, arabinoxylan is associated with processing problems, where addition of xylanases leads to a reduced wort viscosity, haze formation of the beer, and a small increase in extract yield (Courtin et al., 2009; Debyser et al., 1997). In addition, xylanases reduce the viscosity and high water holding capacity of arabinoxylans, which leads to an increased filtration rate and the prevention of fouling on the filtration membranes (Sørensen et al., 2004).

In the pulp and paper industry xylanolytic enzyme preparations can be of great value in the biobleaching of the pulp (Devries and Visser, 2001). Treating pulp with hemicellulases, most notably xylanases, can reduce subsequent chlorine bleaching requirements. The xylanase-mediated hydrolysis of xylan within the pulp matrix opens the tight association between the cellulose, lignin and hemicellulose sufficiently, allowing a better access of the bleaching chemicals to the residual lignin (Kenealy and Jefferies, 2003; Wong et al., 1997). This not only reduces the costs of chemicals, but also the environment problems caused by the use of chlorine are reduced (Jain et al., 2006).

### **1.1.2 Production of health functional oligosaccharides**

In recent years, there has been an increasing awareness that the human gut microflora plays a critical role in maintaining host health, both within the gastrointestinal tract and the absorption of metabolites (Tuohy et al., 2005). Xylo-oligosaccharides (XOS) generated from the enzymatic hydrolysis of arabinoxylan have been reported for a range of functional food activities like antimicrobial activities of acidic XOS and antioxidant activity of feruloylated oligosaccharides (Moure et al. 2006). However, the main biological property of XOS is their ability to stimulate the growth and development of gastrointestinal microflora and hereby have health-promoting effects in human nutrition especially for arabino xylo-oligosaccharides (AXOS) (Cloetens et al., 2010; Grootaert et al. 2007; Guilloux et al. 2010; Sanchez et al., 2009; Van Craeyveld et al. 2008).

### **1.1.3 *Bacillus subtilis* XynA xylanase**

Cereals such as wheat, durum wheat, barley and rye contain proteinaceous xylanase inhibitors which may affect the functionality of microbial xylanases, notably GH11 xylanases (Debyser et al., 1997; Rouau et al., 1998). Because of the increased significance of microbial xylanases in baking and other cereal processing applications (Courtin and Delcour, 2002; Debyser et al., 1997), the problem of xylanase inhibitors reducing the activity of added microbial xylanases is significant (Courtin et al., 2005; Frederix et al., 2004; Sørensen et al.,

2004; Trogh et al., 2004). In order to counteract this problem, a *Bacillus subtilis* XynA xylanase was recently modified to diminish the xylanase-inhibitor interaction – particularly the xylanase-TAXI (*Triticum aestivum* xylanase inhibitor) interaction (Sørensen and Sibbesen, 2006). The D11F/R122D variant is now used commercially, sold as GRINDAMYL™ POWERBake (Danisco A/S, Brabrand, Denmark). This enzyme together with the wildtype has been used as the primary enzyme for the experimental work in this Ph.D. thesis.

#### 1.1.4 Hypotheses

Based on a number of hypotheses, the project will study the enzymatic modification and degradation of different xylan structures by use of selected individual xylanases in order to obtain xylo-oligosaccharides with a uniformed structure. For this purpose the engineered TAXI resistant variant of the *Bacillus subtilis* XynA xylanase (BsX<sub>mut</sub>) and the wildtype (BsX) was used as the primary enzymes under study. The overall hypothesis behind the project is that provision of a better quantitative understanding of the modification and degradation of xylan is a prerequisite for optimally exploiting enzyme reactions in new food and ingredient processes and for exploiting biomass rationally for production of value added products.

The main scientific hypotheses underlying this Ph.D. thesis work became:

- That there must be a correlation between the extent of substrate substitution and the enzyme catalyzed solubilization of xylan.
- For the xylose release, it is either the substitution extent or the number of non-reducing ends that is rate-limiting for the xylose release of WE-AX.
- That the double mutation D11F/R122D has an effect on the kinetics (initial rate and specificity) of the variant BsX<sub>mut</sub> as opposed to the wildtype (BsX).
- That it is possible to exploit high-performance size exclusion chromatography profiles to obtain a quantitative, or at least semi-quantitative, insight into the evolution of the xylo-oligosaccharides during the BsX catalyzed depolymerization of xylan.
- That flaxseed mucilage can be used as a substrate for the enzyme catalyzed production of xylo-oligosaccharides by BsX.
- That wheat bran is a potential source for the enzyme catalyzed production glucurono-xylo-oligosaccharides (GXOS) from wheat bran.

### 1.1.5 Aims of the study

The aims for the PhD project were:

- To obtain a better understanding of the enzyme kinetics and quantitative phenomena of the hydrolysis of xylan substrates by selected monocomponent enzyme preparations.
- To examine the enzyme kinetics of the arabinoxylan depolymerization by assessing the influence of substitutions or the amount of non-reducing ends.
- To assess the exact influence of the double mutation D11F/R122D on the activity BsX<sub>mut</sub> on various xylan containing substrates and on the enzyme's selectivity towards water-unextractable arabinoxylan (WU-AX) vs. water-extractable arabinoxylan (WE-AX), respectively.
- To assess the heterogenous structure of the substrate matrix and the change occurring during the xylanase catalyzed reaction.
- To evaluate the possibilities for using HPSEC as a quantitative method to assess the xylo-oligosaccharide profiles.
- To evaluate the possibility of obtaining xylo-oligosaccharides from flaxseed mucilage.
- To evaluate the potential of obtaining GXOS from wheat bran.

These aims have been fulfilled through 4 papers and 1 report. In Paper 1 (Chapter 2), the kinetics and degree of synergy between the BsX<sub>mut</sub>, a  $\beta$ -xylosidase and two different arabinofuranosidases were examined on WE-AX. The specificity and substrate selectivity of the same xylanase on different xylan containing substrates were assessed in Paper 2 (Chapter 3) in order to obtain an in depth understanding of the mode of action of this xylanase compared to BsX. As a tool for this characterization, a new method was developed, where HPSEC was used to quantify both the depolymerization of the substrate and the release of xylo-oligosaccharides as a result of the xylanase activity (Paper 3, Chapter 4). Based on a characterized xylanase, this enzyme was used for the production of xylo-oligosaccharides with a uniform structure. Since the arabinoxylan from flaxseed had an interesting structure, this substrate was used as potential substrate for BsX in Paper 4 (Chapter 5). For the proof of concept, wheat bran was used as substrate for BsX<sub>mut</sub> with the specific aim of producing GXOS (Chapter 6).

## 1.2 Xylan

### 1.2.1 Plant cell wall

Plant cell wall polysaccharides are the most abundant organic compounds found in the nature. They make up to 90% of the plant cell wall and can be divided into three groups: Cellulose, hemicellulose, and pectin (Carpita 1996). Cellulose represent the major constituent of the cell wall polysaccharides and consists of a linear polymer of  $\beta$ -1,4-linked D-glucopyranosyl residues. The cellulose polymers are present as ordered structures (fibers) and their main function is to ensure the rigidity of the plant cell wall (Burton et al., 2010).

Hemicelluloses are a group of non-cellulosic polysaccharides that have a considerable more complex structure than cellulose. This group includes xyloglucans and xylans and is the second most abundant organic structure in the plant cell wall, accounting for approximately one-third of all renewable organic carbon on earth (Prade 1997). The major hemicellulose polymer in cereals and hardwood is xylan which is a major structural polysaccharide in plant cells (Collins et al., 2005).

Pectins form another group of highly heterogeneous and branched polysaccharides which are rich in D-galacturonic acid residues. The basic structure consists of “smooth” regions of homogalacturonan, and “hairy or ramified regions” of xylogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and polysaccharides compromised mostly of neutral sugars, such as arabinan, galactan, and arabinogalactan (Wong 2008). The composition and chemical structures of these individual segments may vary significantly depending on the origin of the pectin (Huisman et al., 2001).

The hemicelluloses and pectin polysaccharides, as well as the aromatic polymer lignin, interact with the cellulose fibrils, creating a rigid structure strengthening the plant cell wall. They also form covalent cross-links, which are thought to be involved in limiting cell growth and reducing cell wall biodegradability (Devries and Visser, 2001).

### 1.2.2 Structure of xylan

The structure of xylan found in cell walls of plants can differ greatly depending on its origin and the procedure used in its extraction (Joseleau et al., 1992). However, it always consists of a backbone of  $\beta$ -1,4 linked D-xylopyranosyl residues and the xylopyranosyl generally represent more that 50% of the constitutive sugars of the xylan (Saulnier et al., 2007). The xylopyranosyl units can be substituted with L-arabinofuranosyl, 4-O-methyl glucopyranosyl uronic acid residues or they can be esterified with acetic acid. Furthermore, the arabinofuranosyl side chain residues can be esterified with ferulic and p-coumaric acid (de Vries and Visser, 2001) (Figure 1).



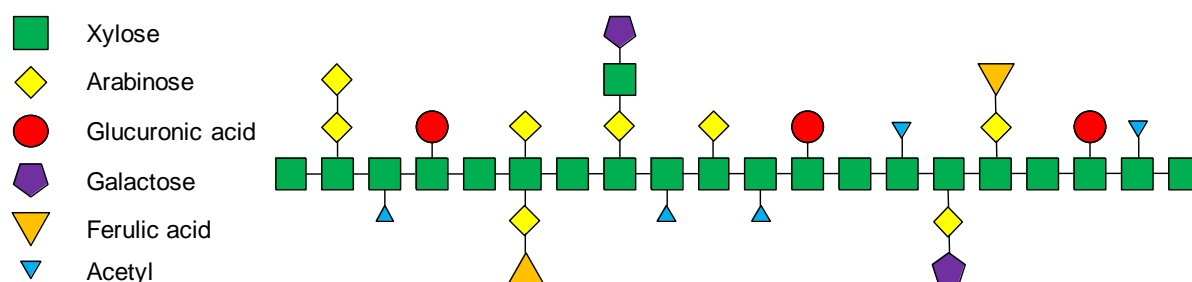


Figure 1. Schematic presentation of xylan (Devries and Visser, 2001). The xylopyranosyl residues in the xylan backbone are  $\beta$ -1,4 linked, whereas the xylopyranosyl, arabinofuranosyl, glucopyranosyl uronic acid and galactopyranosyl substituents are  $\alpha$ -1,2- and/or  $\alpha$ -1,3-linked. Ferulic acids are estrified to the C(O)-5 of the arabinofuranosyl units, whereas acetyl substitutions are estrified to the xylopyranosyl residues in the xylan backbone. In dicots specially, the glucuronic acid is methylated.

In general, xylans present in the main group of plants, the Angiosperms (flowering plants), can be divided in two groups according to whether the xylan is present in Monocotyledons or Dicotyledons.

Xylans present in the cell walls of monocotyles (grasses and cereals) contain large quantities of L-arabinofuranosyl and are therefore often referred to as arabinoxylans. Arabinofuranosyl is linked to the backbone of xylan via an  $\alpha$ -1,2- or  $\alpha$ -1,3-linkage either as single substituents at either the C(O)-2 or C(O)-3 or be di-substituted at both the C(O)-2 and the C(O)-3 position (Gruppen et al., 1993). The xylopyranosyl backbone can also be substituted with  $\alpha$ -D-glucopyranosyl uronic acid or its 4-O-methyl derivative at the C(O)-2 position (Brillouet et al., 1982; Shibuya and Iwasaki, 1985). Furthermore, few publications have also described the presence of O-acetyl substituents (Ishii, 1991; Wende and Fry, 1997). Besides these single units substituents, a variety of di- and trimeric side chains have been identified as minor constituents of arabinoxylan. These side-chains can be composed of arabinofuranosyl only, or can include xylopyranosyl and galactopyranosyl as well (Saulnier et al., 1995; Wende and Fry, 1997).

A few percent of ester-linked hydroxycinnamic acid residues, such as coumaric and ferulic acids, are also found linked to some of the arabinofuranosyl residues (Saulnier et al., 1995; Wende and Fry, 1997). Because they can act as cross-linking agents between polysaccharides, or between polysaccharides and lignin, ferulic acids contribute to wall assembly, promoting tissue cohesion and restricting cell expansion (Saulnier et al., 2007). Although the ferulic acids are an important structural element of arabinoxylan, the amount linked to arabinoxylan is very low and represents 0.2-0.4% of WE-AX (w/w) and 0.6-0.9% of WU-AX in wheat (Bonnin et al., 2000). This corresponds to about 2-4 ferulic acid residues per 1000 xylose residues in WE-AX (6-10 for WU-AX). The ferulic acids can be linked together by ester bonds and these dehydrodiferulic acids are present as substitutions as well, and they are generally linked to the C(O)-5 position of terminal arabinofuranosyl units

(Lequart et al., 1999). Dehydrodiferulic acids were also detected in low amount (10-15 times less than ferulic acid) in WE-AX from wheat (Dervilly-Pinel et al., 2001). For WU-AX, the amount of dehydrodiferulic acids is only 4 times lower than ferulic acid (Lempereur et al., 1998).

The substituents are not always regularly distributed on the arabinoxylan backbone. Particularly for arabinoxylan with high amounts of substituents, an alternation of highly branched and less branched regions has been proposed (Gruppen et al., 1993). Generally cereal endosperm cell walls contain highly branched arabinoxylan, whereas more highly lignified tissues, such as grasses, straw, husks and corncobs, usually contain less branched arabinoxylan.

In contrast to the xylan from monocotyledons, xylan from dicotyledons (hard woods, herbs and woody plants) is an O-acetylated 4-O-methyl- $\alpha$ -D-glucuronoxylan almost without any arabinofuranosyl substitutions. Hardwood xyans are often referred to as glucuronoxylan due to the relatively large amount of D-glucopyranosyl uronic acid attached to the backbone (Devries and Visser, 2001). On average, every tenth xylopyranosyl residue carries an  $\alpha$ -4-O-methylglucopyranosyl uronic acid residue substituted at the C(O)2 position (Reicher et al., 1989). O-acetyl substituents can be located at the C(O)-2 and/or the C(O)-3 position of the xylopyranosyl residues present. Approximately 60-70% of the xylopyranosyl units carries an O-acetyl group in glucuronoarabinoxylan (Teleman et al., 2002). Besides differences in the substitution the degree of polymerization of hardwood xylan (150-200) is higher than that of softwoods (70-130) (Kulkarni et al., 1999).

The different substitution pattern between hard- and softwoods also leads to different physico-chemical properties since side-chains determine the solubility, physical conformation and reactivity of the xylan molecule with other hemi-cellulosic components. Endospermic arabinoxylan of annual plants are therefore more soluble in water and dilute alkali than xylan of lignocellulosic materials due to their branched structures (Ferreira-Filho, 1994). The water-extractable or water-unextractable nature of the (arabino)xylan is also caused by their incorporation in the cell wall structure. While WU-AX are mainly retained in the cell wall by covalent and non-covalent interactions with other neighboring (arabino)xylan molecules and other cell wall constituents such as protein, lignin, cellulose or  $\beta$ -glucan, WE-AX are thought to be loosely bound at the surface of these cell walls (Courtin and Delcour, 2001).

### 1.3 Enzymatic degradation of xylan

Due to the complexity and the heterogeneity of the xylan structure, the enzyme system needed for complete hydrolysis requires both side-group cleaving and depolymerizing enzyme activities. The structural characteristics of the xylan will however influence the composition of the xylanolytic system needed. Enzymatic cleavage of the side chains acquires action of several accessory enzyme activities including  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55), feruloyl esterases (EC 3.1.1.73),  $\alpha$ -glucuronidases (EC 3.2.1.139), and acetyl xylan esterases (EC 3.1.1.72). Depolymerizing activities include endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) that generate unsubstituted or branched xylo-oligosaccharides, including xylobiose and xylotriose, and  $\beta$ -xylosidases (EC 3.2.1.37) that cleave xylobiose and attack the non-reducing ends of short chain xylo-oligosaccharides to liberate xylose (Biely, 1985; Coughlan and Hazlewood, 1993) (Figure 2).

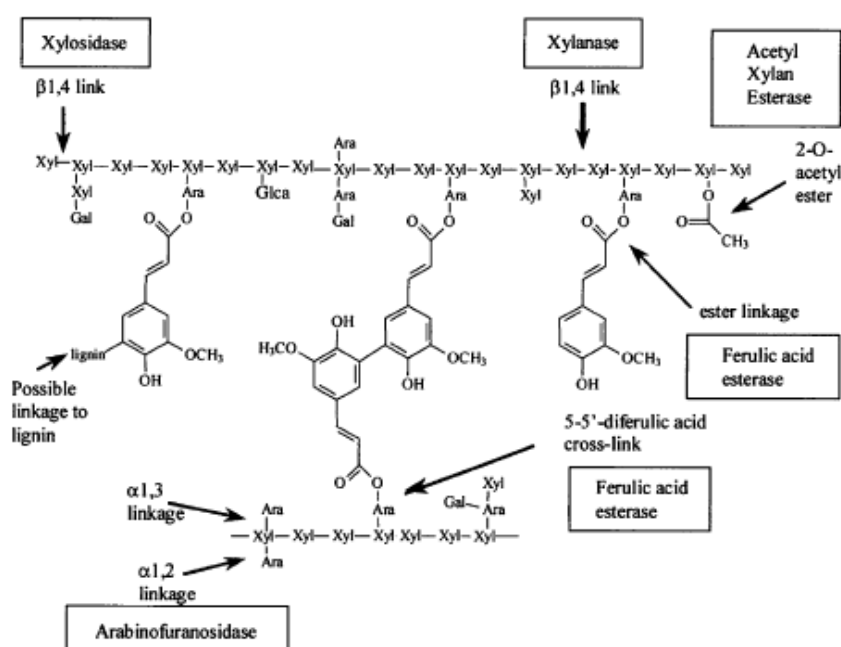


Figure 2. Schematic depiction of the main structural features considered present within WE-AX. The diagram indicates the linkages cleaved by a variety of enzymes that are active against arabinoxylans (Adams et al., 2004).

Several types of synergism have been recognized among the arabinoxylan hydrolyzing enzymes. The most important type concerns the cooperativity between the endo-xylanases attacking the main-chain and the enzymes liberating side chain substituents, such as arabinofuranosyl, feruloyl and acetyl residues, but synergistic interactions also occur among side-chain cleaving enzymes (de Vries et al., 2000; Sørensen et al., 2006, 2007).

### 1.3.1 Endo-1,4- $\beta$ -xylanases

Endo-1,4- $\beta$ -xylanases (xylanases, EC 3.2.1.8) are produced by organisms like bacteria, algae, fungi and plants. These enzymes catalyze the hydrolysis of the  $\beta$ -1,4 linkages in the arabinoxylan backbone, generating a mixture of xylo-oligosaccharides (Biely, 1985). The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases that differ in their physic-chemical properties, structures, mode of actions and substrate specificities (Berrin and Juge, 2008). Within the carbohydrate active enzymes (CAZy) database, xylanase sequences have been reported for glycosyl hydrolase (GH) families 5, 7, 8, 10, 11, 16, 26, 43, 52 and 62 (<http://www.cazy.org/CAZY>; Cantarel et al., 2009). However, only the xylanases classified in GH families 5, 7, 8, 10, 11 and 43 comprise truly distinct catalytic domains with a demonstrated endo- $\beta$ -1,4-xylanase activity. Despite their occurrence in GH families 5, 7, 8 and 43, by far the most xylanases are categorized in family 10 and 11. Whereas fungi and bacteria produce xylanases from both families, all plant xylanases identified to date belong to GH family 10 (Bonnin et al., 2005). The molecular mass of GH10 xylanases is >30 kDa, whereas GH11 xylanase are approximately 20 kDa.

Enzymes from both GH10 and GH11 families cleave the glycosidic bonds by acid-base assisted catalysis using a double displacement mechanism, in which the two catalytic glutamates function as nucleophile and acid-base residues, respectively (Vandermarliere et al., 2008). Despite the same cleavage mechanism, GH10 and GH11 xylanases possess distinct catalytic properties. The GH11 family is monospecific, that is, it consists only of “true xylanases” displaying exclusive substrate specificity towards D-xylopyranosyl containing substrates. Therefore, these enzymes have a lower catalytic versatility than GH10 (Collins et al., 2005).

The differences in catalytic properties can be related to differences in the structure of the two families. Members of GH 10 typically display an ( $\alpha/\beta$ )<sub>8</sub> barrel fold, where the structure has been likened to a “salad bowl” (Biely et al., 1997) (Figure 3A). All reported structures of GH 11 xylanases have been described as a partially closed right hand consisting of only one domain folding into two  $\beta$ -sheets, which form a cleft on which the active site is situated (Collins et al., 2005) (Figure 3.B). The cleft-shaped active site of GH 11 xylanases results in a restricted catalytic behavior where substituents or  $\beta$ -1,3 linkages represent a hindrance to the enzymes' activity (Biely et al., 1997). GH11 xylanases show higher affinity towards a larger number of unsubstituted consecutive xylopyranosyl units. Thus, this type of xylanases are most active on long chain xylo-oligosaccharides, where unsubstituted regions of the arabinoxylan backbone are preferentially cleaved (Berrin and Juge, 2008). The active site of GH10 xylanases is a shallow groove which is reflected in a specificity towards a lower number of unsubstituted consecutive xylopyranosyl units. This group of enzymes is less

hampered by the presence of 4-O-methyl-D-glucopyranosyl uronic acid, acetate and arabinofuranosyl substituents along the xylan backbone and can therefore act near the substitutions and cleave decorated regions (Biely et al., 1997).

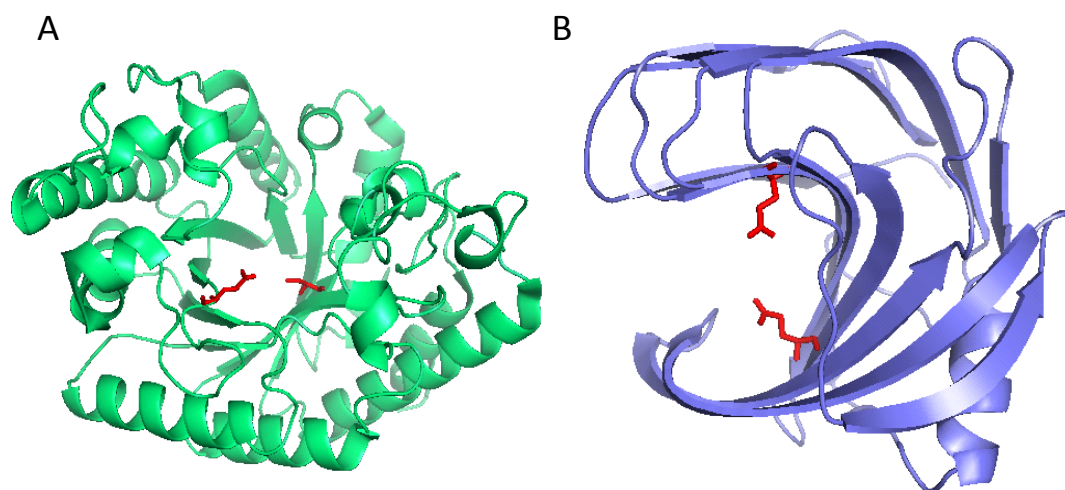


Figure 3. Overall structure of (A) GH10 *Cellvibrio mixtus* xylanase (PDB 1UQY) (green) and (B) GH11 *B. subtilis* xylanase (PDB 2B45). The catalytic residues of both enzymes are highlighted in red (*C. mixtus*: Glu157 and Ser262; *B. subtilis*: Glu78 and Glu172). These figures were drawn using PyMOL (v0.99) (DeLano Scientific, San Carlos, CA)

The slightly diversity between the catalytic sites of GH 10 and 11 enzymes result in small differences in their hydrolytic end-products (Fujimoto et al., 2004; Pell et al., 2004; Pollet 2010). For GH11 xylanases, the main end products from the hydrolysis are unsubstituted xylo-oligosaccharides like xylobiose and xylotriose, which can be related to the ability of hydrolyzing unsubstituted regions of xylan (Berrin et al., 2007; Biely et al., 1997; Kormelink et al., 1993). The xylo-oligosaccharides resulting from the activity of GH10 xylanases are shorter than those produced by GH11 xylanases, and GH10 xylanases are able to release xylose as end product (e.g. Charnock et al., 1998; Collins et al., 2005). Furthermore, the formed oligosaccharides by GH10 xylanases carry substituents at the nonreducing terminal of the  $\beta$ -1,3-xylopyranosyl residue (Biely et al., 1997; Maslen et al., 2007).

Xylanases change the structure and physicochemical properties of arabinoxylan: On one hand, they solubilise WU-AX which leads to loss in water-holding capacity and, on the other hand, they degrade WE-AX, which leads to a decrease in viscosity (Courtin et al., 2001). GH10 xylanase are preferentially more active against WE-AX, whereas GH11 xylanases are more active against WU-AX (Biely et al., 1997, Courtin and Delcour, 2001; Maes et al., 2004). The higher activity towards WU-AX might be explained by the smaller size of the GH11 xylanase (~20 kDa) than the GH10 xylanases (>30 kDa), which make it easier to penetrate the cell-wall network in e.g. wheat bran (Beaugrand et al., 2004).

Xylanases from *Bacillus circulans* (Ludwiczek et al., 2007), *B. subtilis* and *A. niger* (Vandermarliere et al., 2008) have a secondary substrate-binding site on the surface of the finger region of the catalytic domain. This binding site enhances the activity of the enzyme on heteroxylan, and might have a function similar to that of the carbohydrate binding modules (CMB) of other glycoside hydrolases (Ludwiczek et al., 2007). Furthermore, it has been suggested that this secondary substrate-binding site may play a role in directing the substrate selectivity of these xylanases towards either WE-AX or WU-AX (Berrin and Juge, 2008).

Specific for GH11 xylanase, studies have shown that the thumb-like lobe can move, and thus the width of the active site cleft can be regulated (Murakami et al., 2005). This flexibility plays a crucial role in the binding of the substrate and the release of products from the active site (Connelly et al., 2000, Pollet et al., 2009). Therefore, the thumb-like lobe is essential for the hydrolytic activity of these xylanases.

### **1.3.2 $\beta$ -Xylosidases**

$\beta$ -Xylosidases (EC 3.2.1.37) catalyze the cleavage of xylobiose and the hydrolytic liberation of the terminal xylopyranosyl unit from the non-reducing end of xylo-oligosaccharides arising from the xylanase activity (Rasmussen et al. 2006). Enzymes exhibiting  $\beta$ -xylosidase activity are categorized into eight different GH families: 3, 30, 39, 43, 52, 54, 116 and 120 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Certain bifunctional  $\beta$ -xylosidases may also exert arabinofuranosidase activity (and vice versa) – those are mainly categorized in GH family 3 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). The affinity of  $\beta$ -xylosidases decreases with increasing chain length of the xylo-oligosaccharides (Rasmussen et al., 2006). Several  $\beta$ -xylosidases have also been reported to also attack polymeric xylan in an exo fashion (Biely et al., 2000).

### **1.3.3 $\alpha$ -L-Arabinofuranosidases**

$\alpha$ -L-Arabinofuranosidases (arabinofuranosidase, EC 3.2.1.55) catalyze the hydrolysis of non-reducing  $\alpha$ -L-arabinofuranosyl residues from the xylopyranosyl residues in arabinoxylan. Based on their amino acid sequence, the arabinofuranosidases are categorized into several GH families: 3, 43, 51, 54 and 62 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Only the sequences classified in families 43, 51, 54, and 62 contained truly distinct catalytic domains with a demonstrated arabinofuranosidase activity. The sequences reported on families 3, and 10 were polyspecific, containing two catalytic domains that belonged to different GH families (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Although the arabinofuranosidases are very specific for arabinoxylans, their mode of action on arabinoxylan and arabinoxylan-derived oligosaccharides are different. Arabinofuranosidases catalyze the hydrolysis of  $\alpha$ -1,2,

$\alpha$ -1,3 and even  $\alpha$ -1,5 glycosidic bonds of  $\alpha$ -L-arabinofuranosyl residues (De Vries and Visser, 2001).

An arabinofuranosidase which specifically catalyses the release of  $\alpha$ -1,3-L-linked arabinofuranosyl substituents from doubly substituted xylose residues in arabinoxylan has been cloned from *Bifidobacterium adolescentis* (Van den Broek et al., 2005; Van Laere et al., 1997). This enzyme has been assigned to GH family 43 (<http://www.cazy.org/CAZY>). An arabinofuranosidase from *Aspergillus awamori* which apparently catalyses the release of both  $\alpha$ -1,2- and  $\alpha$ -1,3-L-linked arabinofuranosyls on single substituted xyloses in arabinoxylans was identified already in 1993 (Kormelink et al., 1993). More recently, a fungal enzyme from *Humicola insolens*, categorized as a GH family 43 enzyme, was also found able to catalyze the removal of  $\alpha$ -1,3-L-linked arabinofuranosyl substituents from doubly substituted xylose in arabinoxylan (Sørensen et al., 2006). In contrast, the majority of the other fungal arabinofuranosidases, notably those from *Aspergillus* spp. catalyze the release of  $\alpha$ -1,3-L-linked arabinofuranosyl substituents from singly substituted xylose and are categorized in GH families 51, 54, and 62.

#### 1.3.4 Enzymes acting on other substituents

$\alpha$ -1,2-Glucuronidases (glucuronidase, EC 3.2.1.131) hydrolyze the linkage between the (4-O-methyl)- $\alpha$ -D-glucopyranosyl uronic acid substituent and the  $\beta$ -D-xylopyranosyl unit. Glucuronidase activity is found in bacteria and fungi (Puls et al., 1987). Some glucuronidases are able to act on polymeric xylan and but most glucuronidases are active on oligosaccharides (Khandke et al., 1989; Tenkanen and Siika-aho, 2000). An interesting question related to the substrate specificity of glucuronidases is, whether the enzyme is specific for glucopyranosyl uronic acid or 4-O-methyl-D-glucopyranosyl uronic acid. The enzyme from *A. niger* catalyzed the hydrolysis of GlcAXyl<sub>3</sub> about two times faster than MeGlcAXyl<sub>3</sub> (Ushida et al., 1992). This example suggests that the 4-O-methyl group in the acid is not decisive for the action of the enzyme.

Acetyl groups are removed by acetyl xylan esterases (EC 3.1.1.72), which cleave the acetyl groups from positions C(O)-2 and C(O)-3 of  $\beta$ -D-xylopyranosyl units (Biely, 1985; Kormelink and Voragen, 1993). Acetyl xylan esterases show great diversity and have been assigned to seven carbohydrate esterase families: 1, 2, 3, 4, 5, 6, and 7 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Feruloyl esterases (EC 3.1.1.73) catalyzes the hydrolysis of the ester linkages between ferulic or p-coumaric acids from an esterified sugar, which is usually arabinofuranose in a wide range of substrates (Smith and Hartly, 1983). This group of enzymes has been classified into carbohydrate esterase family 1 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009).

## 1.4. Xylanase inhibitors

Cereals such as wheat, durum wheat, barley and rye contain proteinaceous xylanase inhibitors which may affect the functionality of microbial xylanases, notably GH11 xylanases (Debyser et al., 1997; Rouau et al., 1998). Parameters such as wheat variety, milling and growing conditions determine the xylanase inhibitor concentration in wheat (Bonnin et al., 2005). The actions of xylanase inhibitors profoundly impact the functional properties of arabinoxylan in cereal processing such as gluten-starch separation and bread making, where the activity of the xylanases is reduced. Furthermore, the inhibitors themselves can have an impact on the biotechnological application through their action directly on the arabinoxylan population (Juge and Svensson, 2006).

By now, three distinct types of xylanase inhibitors have been identified in cereals: TAXI (*Triticum aestivum* xylanase inhibitor), XIP (xylanase-inhibiting protein) and TL-XI (thaumatin-like xylanase inhibitor) (Croes et al., 2008). These classes show a large structural variety leading to different modes and specificities of inhibition (Pollet et al., 2009).

### 1.4.1 TAXI

TAXI are proteins of ~40 kDa that specifically inhibit bacterial and fungal GH11 xylanases (Gebruers et al., 2001) (Figure 4). Based on immunoblot quantification of xylanase inhibitors in eight wheat cultivars, whole meal contains, on average, 130 mg kg<sup>-1</sup> TAXI (Croes et al., 2009). Inhibitor concentrations in wheat flour can vary up to five-fold depending on milling fraction or variety (Bonnin et al., 2005). The inhibition by TAXI is complicated by the presence of two distinct forms, TAXI-I and TAXI-II, which share 86% sequence identity, but differ in pI (8.9 and 9.3, respectively) (Gebruers et al., 2001). The two inhibitors also differ in specificities, i.e. not all xylanases are inhibited by both TAXI-I and TAXI-II (Gebruers et al., 2004). In addition, the type of inhibition (competitive/noncompetitive) varies depending on the xylanase (Berrin and Juge, 2008). For example, competitive inhibition was reported for *P. funiculosus* xylanase C (Furniss et al., 2002), whereas the *B. subtilis* xylanase was non-competitively inhibited by both TAXI proteins (Berrin and Juge, 2008).



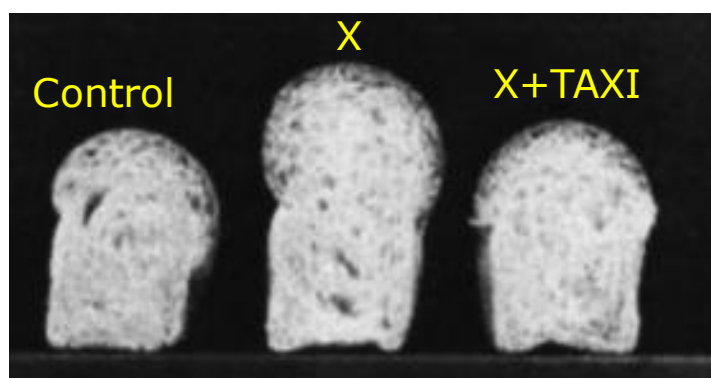


Figure 4. Effect of xylanase (X) and *Triticum aestivum* xylanase inhibitor (TAXI) on bread volume (Debyser et al., 1999).

Based on structures of TAXI in complex with different GH 11 xylanases, it was shown that TAXI directly interacts reversibly with the active site region of the enzyme. This inhibition is caused by the binding of several amino acid residues of TAXI to the active site cleft of the enzyme. This binding prevents the access and binding of the xylan substrate in the active site (Bourgois et al., 2007; Sansen et al., 2004). The structure of a TAXI-I-*A. niger* xylanase complex also revealed a substrate-mimicking contacts, where the binding subsites fill the whole substrate-docking region (Sansen et al., 2004).

Site-directed mutagenesis of 22 surface residues surrounding the active site cleft of *B. subtilis* XynA xylanase, has indicated that residues on “finger IV”, more specifically D11, play an important role in the interaction with TAXI-I (Sørensen and Sibbesen, 2006) (Figure 5). Mutations of D11 into different acids completely abolished the interaction between the xylanases and the inhibitor. Also site-directed mutations of D37 of a *A. niger* xylanase completely abolished TAXI-mediated inhibition of this GH11 xylanase (Tahir et al., 2004). This also indicated the importance of the C-terminal end for observed differences in xylanases specificity among different TAXI-type inhibitors (Raedschelders et al., 2005; Sansen et al., 2004). However, the both variants also showed significant decreases in enzyme activity, most likely due to hampered substrate binding and hydrolysis. Recently, a highly active TAXI-insensitive XynA variant was created, in which replacement of G12 by tryptophane led to sterical hindrance of the docking of TAXI onto the enzyme without, impeding substrate binding and hydrolysis (Bourgois et al., 2007).

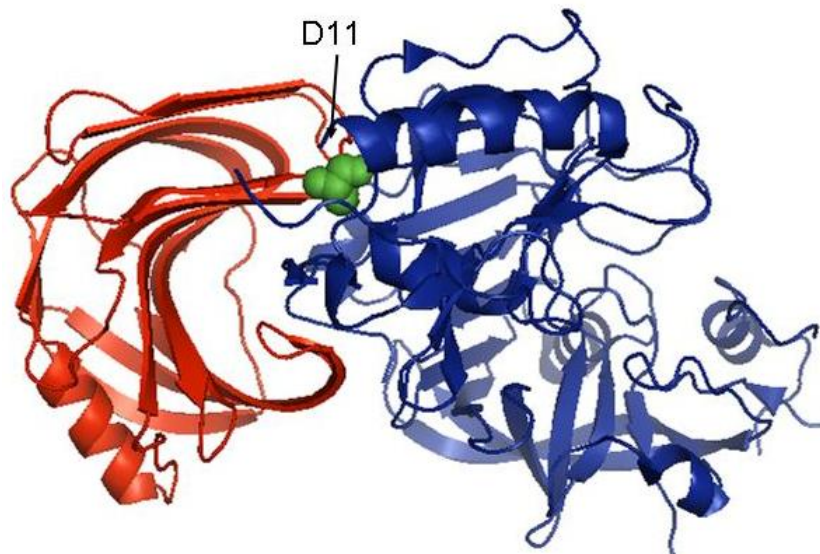


Figure 5. Structure of TAXI (blue) in complex with the *B. subtilis* XynA xylanases (red) (PDB 2B42). The residue D11 of the xylanases is enhanced (green). This figure was drawn using PyMOL (v0.99) (DeLano Scientific, San Carlos, CA).

#### 1.4.2 XIP

XIP is a glycosylated monomeric protein with a molecular mass of 29 kDa and pI values of 8.7-8.9 (Durand et al., 2005; Goesaert et al., 2005; Juge et al., 2004; McLauchlan, 1999). XIP protein levels in whole meal from eight cultivars ranged from 156 to 371 mg kg<sup>-1</sup>, with an average value of 235 mg kg<sup>-1</sup> making it the most abundant xylanase inhibitor in wheat grain (Croes et al., 2009). Kinetic studies have established that XIP is a strong inhibitor of fungal GH10 and GH11 xylanases, but it does not inhibit any of the bacterial xylanases tested (Flatman et al., 2002). XIP has two independent and opposite enzyme-binding sites, allowing binding of two glycoside hydrolases that display a different fold (Payan et al., 2004). The inhibition is competitive with a 1:1 stoichiometry and involves substrate-mimetic contacts in which the inhibitor blocks the active site (Payan et al., 2004; Tahir et al, 2002). However, not all GH10 and GH11 xylanases are inhibited by XIP, and the reasons for the lack of inhibition of these xylanases differ with the GH family origin of the enzymes. For GH11 xylanases, the resistance to XIP can, to a large extent, be explained by the structural determinants around the “thumb” region of the enzyme (Payan et al., 2004). GH11 xylanases with amino acid insertions in the tip region of the “thumb” are more likely to be uninhibited by XIP (Belien et al., 2007; Berrin et al., 2007; Payan et al., 2004). For GH10 xylanases, the lack of inhibition can, at least partly, be due to differences in the loop regions located around the active site (Payan et al., 2004).

### 1.4.3 TL-XI

Recently, a third type of xylanase inhibitor, thaumatin-like xylanase inhibitor protein (TL-XI), was identified in wheat (Fierens et al., 2007). Among the known proteinaceous xylanase inhibitors, they have the smallest molecular mass (~18 kDa) and occur on average in the smallest amounts in whole meal of diverse wheat cultivars (~ 100 mg kg<sup>-1</sup>) (Croes et al., 2009). In contrast to TAXI and XIP, TL-XI was found to exert its inhibition in a non-competitive way (Fierens et al., 2007). The TL-XI inhibits a number of fungal and bacterial GH11 xylanases, but is inactive towards GH10 xylanases tested so far.

Common for all three types of xylanase inhibitors are, that binding of the inhibitors directly to arabinoxylan has been described (Fierens et al., 2008). Furthermore, all three inhibitors have a tendency to bind to WU-AX, but not WE-AX. The affinity of the xylanase inhibitors for the (arabino)xylans generally increase with decreasing A/X ratios. This means that the interactions between the inhibitor and the xylan chains manifest themselves more, when only a low degree of – or no – substitutions are present on the xylan. This trend was most clear with the insoluble arabinoxylan. As a result, the interaction of these inhibitors with arabinoxylan may also affect the substrate selectivity of xylanases (Berrin and Juge, 2008).

Since the significance of microbial xylanases in baking and other cereal processing applications has increased (Courtin and Delcour, 2002; Debyser et al., 1997), the problem of xylanase inhibitors reducing the activity of added microbial xylanases is significant (Courtin et al., 2005; Frederix et al., 2004; Sørensen et al., 2004; Trogh et al., 2004). Together with specificity, pH and thermostability, the insensitivity towards xylanase inhibitors is thus an important xylanolytic property, when considering the use of xylanases in biotechnological applications, notably cereal based applications. Therefore, it is of considerable interest to engineer the insensitivity of the xylanases.

In order to counteract this problem, a *B. subtilis* XynA xylanase (BsX) was modified to diminish the xylanase-inhibitor interaction – particularly the xylanase-TAXI interaction (Sørensen and Sibbesen, 2006). Biochemical studies in combination with protein structural information have significantly contributed to the knowledge of the hydrolytic activity of this xylanases and to the role of individual amino acids. Nevertheless, information about the enzymatic modification and degradation of different xylan structures in order to obtain xylo-oligosaccharides with a uniformed structure by this particular enzyme is still missing.

## Chapter 2 Endo-1,4- $\beta$ -xylanase activity is rate-limiting for enzymatic xylose liberation from soluble wheat arabinoxylan

This chapter is extended in the form of Paper 1:

Endo-1,4- $\beta$ -xylanase activity is rate-limiting for enzymatic xylose liberation from soluble wheat arabinoxylan

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### 2.1 Key points

In the degradation of arabinoxylan to monomeric sugars, several different enzymes are needed (Tenkanen 1996). Arabinofuranosyl side groups are mainly cleaved off by arabinofuranosidases and the backbone is randomly hydrolyzed to short xylo-oligosaccharides by xylanases. The xylo-oligosaccharides are further hydrolyzed to xylopyranosyl by  $\beta$ -xylosidases. Synergism has been recognized among the arabinoxylan hydrolyzing enzymes, where the most important type concerns the cooperativity between the xylanases attacking the main-chain and the arabinofuranosidase liberating the arabinofuranosyl side chain substituents (Sørensen et al., 2003). In this study two arabinofuranosidases have been used: Arabinofuranosidase from *Aspergillus niger* (AF<sub>An</sub>) which catalyze the release of (1 $\rightarrow$ 3)- $\alpha$ -L-linked arabinofuranosyl substituents from singly substituted xylopyranosyl and an arabinofuranosidase from *Bifidobacterium adolescentis* (AF<sub>Ba</sub>), which specifically catalyses the release of (1 $\rightarrow$ 3)- $\alpha$ -L-linked arabinofuranosyl substituents from doubly substituted xylopyranose residues in arabinoxylan (Van den Broek et al., 2005; Van Laere et al., 1997) (Figure 6)

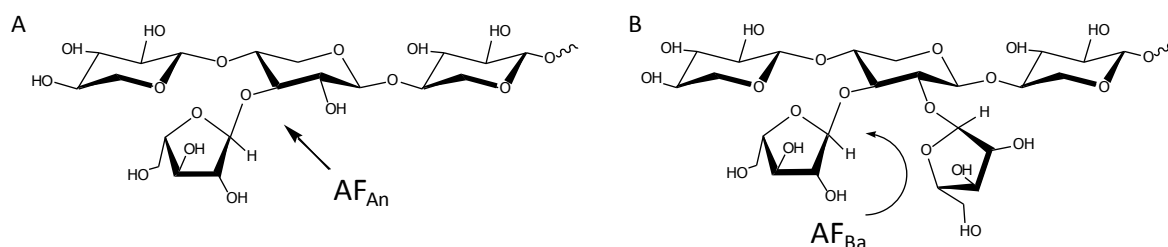


Figure 6. Structural elucidation of the release of arabinofuranosyl substitution from xylotriose catalyzed by arabinofuranosidases from (A) *Aspergillus niger* (AF<sub>An</sub>) and (B) *Bifidobacterium adolescentis* (AF<sub>Ba</sub>) (Van den Broek et al., 2005; Van Laere et al., 1997).

The discovery of such highly specific enzymes raises the possibility of examining, what is most important for a fast hydrolysis in respect to the liberation of xylopyranosyl from WE-AX.

Is it the removal of the arabinofuranosyl substituents by the arabinofuranosidases or the increase in available non-reducing ends by the xylanases. In the present work WE-AX was treated with different mixtures of xylanase,  $\beta$ -xylosidase,  $AF_{An}$  and  $AF_{Ba}$ . Furthermore, the kinetic consequences of the specificity of arabinofuranosidase attack, i.e. removal of arabinofuranosyl from singly or doubly substituted xyloses, in relation to synergism and enzyme catalyzed depolymerization of the xylan backbone was investigated.

## 2.2 Conclusion

The hypothesis for this work was that, it was either the substitution extent or the number of non-reducing ends that was rate-limiting for the xylopyranosyl release. This was investigated based on the enzyme catalyzed hydrolysis of WEAX by the cooperative action of xylanase,  $\beta$ -xylosidase and arabinofuranosidases having different specificities. The results demonstrated that the xylanase activity is the dominating rate-limiting enzyme activity for the xylopyranosyl release. The consequence of the different arabinofuranosidase specificity on the catalyzed hydrolysis of arabinoxylan was that although the  $AF_{Ba}$  had higher rate constant on the arabinoxylan, the  $AF_{An}$  exhibited better synergy with  $\beta$ -xylosidase to release xylose. However, the synergistic effect of the  $AF_{An}$  with  $\beta$ -xylosidase on xylose release was lower than the synergy between the xylanase and  $\beta$ -xylosidase. These results confirmed the hypothesis that there is correlation between the extent of substrate substitution and enzymatic solubilization of xylan. A particular feature was that the provision of more (unsubstituted) non-reducing ends resulting from xylanase action was more efficient to boost the  $\beta$ -xylosidase activity, than provision of more (randomly) unsubstituted xyloses in the arabinoxylan backbone by the  $AF_{An}$ .

Both the xylose and the arabinose release rates ( $v$ ) for different enzyme combinations in response to the increase in substrate (arabinoxylan) concentration appeared to follow the classic rectangular hyperbola Michaelis-Menten type curves. Thus, apparent values for the classic kinetic parameters of these reactions could be derived by non-linear regression. This is somewhat surprisingly, since the introduction of  $BsX$  and  $AF_{An}$  *increases* the true substrate concentration for the  $\beta$ -xylosidase. This violates one of the principles underlying Michaelis-Menten kinetics, namely that the initial substrate concentration *decreases* during the reaction. This indicates that it is the attackable linkages that are the substrate for the  $\beta$ -xylosidase and not the single polymer.



## Abstract

This study investigated the kinetics of enzymatic degradation of soluble wheat arabinoxylan by monitoring the release of xylose and arabinose during designed treatments with selected mono-component enzymes at different substrate concentrations. The results of different combinations of  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55), one derived from *Aspergillus niger* (AF<sub>An</sub>) and one from *Bifidobacterium adolescentis* (AF<sub>Ba</sub>), respectively, a  $\beta$ -xylosidase (EC 3.2.1.37) from *Trichoderma reesei*, and an engineered D11F/R122D variant of *Bacillus subtilis* XynA endo-1,4- $\beta$ -xylanase (EC 3.2.1.8) were examined. The selected  $\alpha$ -L-arabinofuranosidases catalyze liberation of arabinose residues linked 1 $\rightarrow$ 3 to singly (AF<sub>An</sub>) or doubly (AF<sub>Ba</sub>) substituted xyloses in arabinoxylan, respectively. The AF<sub>Ba</sub> enzyme catalyzed the release of more arabinose, i.e. had a higher rate constant than AF<sub>An</sub>, when added to arabinoxylan at equimolar levels. With respect to the xylose release, AF<sub>An</sub> - as expected - exhibited a better synergistic effect than AF<sub>Ba</sub> with  $\beta$ -xylosidase. However, the synergistic effects between  $\beta$ -xylosidase and the  $\alpha$ -L-arabinofuranosidases on the xylose release were low as compared to the effect of xylanase addition with  $\beta$ -xylosidase, which increased the xylose release by ~25 times in 30 minutes. At equimolar addition levels of the four enzymes, the xylanase activity was thus rate-limiting for the  $\beta$ -xylosidase catalyzed depolymerization to release xylose from arabinoxylan.

Keywords: arabinoxylan hydrolysis, enzyme kinetics,  $\alpha$ -L-arabinofuranosidase, synergy

## 1. Introduction

The enzymatic hydrolysis of arabinoxylan is a complicated process which requires the action of different enzyme activities for complete degradation. These enzyme activities first and foremost include endo-1,4- $\beta$ -xylanase (xylanase, EC 3.2.1.8),  $\beta$ -xylosidase (EC 3.2.1.37), and  $\alpha$ -L-arabinofuranosidase (arabinofuranosidase, EC 3.2.1.55) activities, but may for certain arabinoxylan substrates include other accessory enzymes, e.g. feruloyl esterase (EC 3.1.1.73) and acetyl esterase (EC 3.1.1.6) [1, 2]. Much attention is currently given to the degradation of arabinoxylan in relation to “second generation” biofuel processes based on conversion of different types of plant biomass [3]. However, nutritional aspects of dietary arabinoxylan and its degradation products with respect to prebiotic activity are also receiving increasing research interest [4, 5]. Some of the structural details of wheat arabinoxylan, which are crucial for rationally designing its enzymatic degradation, are still unclear, but the levels of mono-substitution vs. di-substitution are known. Wheat arabinoxylan thus consists of a backbone of (1,4)- $\beta$ -linked D-xylopyranosyl residues that are partially substituted by single  $\alpha$ -L-arabinofuranosyl residues bound (1 $\rightarrow$ 2) or (1 $\rightarrow$ 3) to xylose, that is singly substituted xyloses, or as (1 $\rightarrow$ 2) and (1 $\rightarrow$ 3) to doubly substituted xyloses [6]. The extent and distribution of  $\alpha$ -L-arabinofuranosyl residues vary significantly depending on the wheat source, e.g. the variety and tissue origin [7]. The presence of these substitutions on the xylan backbone is believed to limit the enzymatic depolymerization of arabinoxylan by hindering the xylanase attack [3, 8]. Similarly, arabinose substituents at terminal or adjacent to terminal, D-xylopyranosyl residues in xylooligosaccharides hinder catalysis by the  $\beta$ -xylosidase from *Trichoderma reesei* [9]. The enzymatic degradation of arabinoxylan can thus be expected to depend on the degree of arabinose substitution, the position and type of the arabinofuranosyl glycosidic bonds, and their distribution along the xylan backbone. A particular issue in the degradation of wheat arabinoxylans is the presence of similarly linked (1 $\rightarrow$ 3)  $\alpha$ -L-



1 arabinofuranosyls that require different arabinofuranosidase activities depending on whether  
 2 the  $\alpha$ -L-arabinofuranosyls are linked to singly or doubly substituted xyloses.  
 3 Based on their amino acid sequence, the arabinofuranosidases are categorized into several  
 4 glycoside hydrolase (GH) families: 3, 43, 51, 54 and 62 (<http://www.cazy.org/CAZY>). These  
 5 enzymes catalyze the hydrolysis of  $\alpha$ -1,2,  $\alpha$ -1,3 and even  $\alpha$ -1,5 glycosidic bonds of  $\alpha$ -L-  
 6 arabinofuranosyl residues [10]. Certain “multifunctional” arabinofuranosidases may also exert  
 7  $\beta$ -xylosidase activity – mainly those categorized in GH family 3. An arabinofuranosidase  
 8 which specifically catalyses the release of (1 $\rightarrow$ 3)- $\alpha$ -L-linked arabinofuranosyl substituents  
 9 from doubly substituted xylose residues in arabinoxylan has been isolated and cloned from the  
 10 gastrointestinal bacterium *Bifidobacterium adolescentis* [11, 12]. This enzyme has been  
 11 assigned to GH family 43 (<http://www.cazy.org/CAZY>). An arabinofuranosidase from  
 12 *Aspergillus awamori* which apparently catalyses the release of both (1 $\rightarrow$ 2) and (1 $\rightarrow$ 3)- $\alpha$ -L-  
 13 linked arabinofuranosyls on single substituted xyloses in arabinoxylans was identified already  
 14 in 1993 [13]. More recently, a fungal enzyme from *Humicola insolens*, categorized as a GH  
 15 family 43 enzyme, was also found able to catalyze the removal of (1 $\rightarrow$ 3)- $\alpha$ -L-linked  
 16 arabinofuranosyl substituents from doubly substituted xylose in arabinoxylan [14]. In contrast,  
 17 the majority of the other fungal arabinofuranosidases, notably those from *Aspergillus* spp.  
 18 catalyze the release of (1 $\rightarrow$ 3)- $\alpha$ -L-linked arabinofuranosyl substituents from singly  
 19 substituted xylose and are categorized in GH families 51, 54, and 62.  
 20  $\beta$ -Xylosidases (EC 3.2.1.37) catalyze the cleavage of xylobiose and attack the non-reducing  
 21 ends of relatively short xylooligosaccharides to liberate xylose [1, 2]. Enzymes exhibiting  $\beta$ -  
 22 xylosidase activity are categorized into the following seven different GH families: 3, 30, 39,  
 23 43, 52, 54, 116 and 120 (<http://www.cazy.org/CAZY>).  
 24 The potential synergistic effects of microbial enzymes acting on arabinoxylan have been  
 25 examined with combinations of arabinofuranosidases from *Aspergillus niger*, *B. adolescentis*,  
 26 *H. insolens*, and *Meripilus giganteus* [11-12, 14],  $\beta$ -xylosidase from *T. reesei* [1, 3, 14-15],

1 and various xylanases from e.g. *Aspergillus aculeatus*, *H. insolens*, *Thermomyces*  
2 *lanuginosus*, and *Bacillus subtilis* [14, 16-17]. Recently optimal, “minimal” mixtures of  
3 xylanase,  $\beta$ -xylosidase and arabinofuranosidase were designed, using the minimal number and  
4 the minimal dosages of the most active enzyme activities in the optimal proportions, to  
5 accomplish efficient degradation of different types of wheat arabinoxylan [17]. However,  
6 with respect to the liberation of xylose, it is unclear whether it is the removal of the arabinose  
7 substituents or the increase in the available substrate sites, i.e. available non-reducing ends,  
8 for  $\beta$ -xylosidase that are most important for fast hydrolysis. The purpose of the current study  
9 was to examine the enzyme kinetics for arabinoxylan degradation to define the rate-limiting  
10 step(s) and in turn provide a better base for designing and controlling the multi-enzyme  
11 catalyzed degradation optimally. A particular objective of this study was to evaluate the  
12 kinetic consequences of the specific arabinofuranosidase attack, i.e. removal of arabinose  
13 from singly or doubly substituted xyloses, in relation to synergism and enzyme catalyzed  
14 depolymerization of the xylan backbone by xylanase and  $\beta$ -xylosidase.

## 16 2. Materials and methods

### 17 2.1. Chemicals and substrate

18 L-Arabinose, D-xylose, *p*-nitrophenyl xyloside (pNPX), and *p*-nitrophenyl arabinoside (pNPA)  
19 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water-soluble wheat  
20 arabinoxylan (WEAX) was purchased from Megazyme (Bray, County Wicklow, Ireland).  
21 The basic characteristics of WEAX are shown in Table 1.

### 23 2.2. Enzymes

24 The arabinofuranosidases from *A. niger* (AF<sub>An</sub>) and *B. adolescentis* (AF<sub>Ba</sub>) were both  
25 purchased from Megazyme (Bray, County Wicklow, Ireland) and used without further  
26 purification. The AF<sub>Ba</sub> is a cloned enzyme, which is indicated on the suppliers' product sheet

to have a molecular weight of 60 kDa and to be from a *Bifidobacterium sp.*. However, references are made to the articles by Van Laere et al. (1997) [11] and Van den Broek et al. (2005) [12] that reported the identification and cloning of the enzyme from *B. adolescentis*. For this reason we refer to this enzyme activity as derived from *B. adolescentis* and as a GH family 43 enzyme. The  $\beta$ -xylosidase from *T. reesei* was purified from Celluclast 1.5 L by Novozymes A/S as described previously [3]. The *Bacillus subtilis* xylanase (BsX<sub>mut</sub>) was obtained from Danisco A/S (Brabrand, Denmark) in a starch powder formulation. This xylanase, BsX<sub>mut</sub>, is an engineered D11F/R122D variant of a *Bacillus subtilis* XynA xylanase. The enzyme carries the Swiss Prot entry P18429, XynA\_BACSU and has been classified as a glycosyl hydrolase family 11 xylanase according to sequence homology (<http://www.cazy.org/CAZY>). The BsX<sub>mut</sub> enzyme is less inhibited by TAXI (*Triticum aestivum* xylanase inhibitor) than the native XynA xylanase [16]; for this present study the BsX<sub>mut</sub> was purified from a Grindamyl H 640 enzyme preparation principally as described by Sørensen and Sibbesen [16]. The pH and temperature optima for the used enzymes are shown in Table 2. Furthermore, each enzyme was essentially pure as shown by SDS PAGE (Supp. 1).

## 2.3 SDS PAGE

Protein content was analyzed by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS–PAGE). The gel type was a Criterion™ XT Precast Gel, 12% Bis-Tris (BIO-RAD, Hercules CA, US). The SDS-Page was run automatically using Power Pac™ HC (BIO-RAD) at 200 V constant for 60 min. The running buffer was XT MOPS (BIO-RAD) and the gel was stained with Bio-Safe™ Coomassie (BIO-RAD) according to the manufacturer's procedure.

## 2.4. Enzymatic hydrolysis of WEAX

2 g·L<sup>-1</sup> WEAX in 1 mL aliquots, were incubated with pre-defined concentrations of different enzymes in 100 mM sodium acetate buffer pH 5 at 40°C. This condition was chosen as a

compromise of the optima of the assessed enzymes (Table 2). Samples were withdrawn after 0, 0.5, 1, 2, and 4 h and heated immediately at 100°C for 10 min. to halt the enzymatic reaction. In the reaction rate study, hydrolysis was performed at different substrate concentrations, i.e. weight of arabinoxylan, ranging from 0.125 g·L<sup>-1</sup> to 4 g·L<sup>-1</sup>. In order to compare rates of enzymatic hydrolysis all enzymes were added at an equal molar concentration of 0.025 nmol · g<sup>-1</sup> WEAX, i.e. at a concentration of ~0.05 nM. After hydrolysis, samples were filtered (0.2 µm filter), and the levels of arabinose and xylose were determined by high performance anion exchange chromatography.

## 2.5. High-performance anion exchange chromatography (HPAEC)

Hydrolysates were applied onto a Dionex BioLC system fitted with a Dionex CarboPac PA1 analytical column (4x250 mm) (Dionex Corp., Sunnyvale, CA, USA). The monosaccharides were separated isocratically with 10 mM NaOH at a flow rate of 1 mL min<sup>-1</sup>. Strongly bound anions were eluted from the column by increasing the NaOH concentration to 0.5 M over a period of 14 min. Before applying the subsequent sample, the NaOH concentration was reduced to 10 mM for 5 min. Monosaccharides were detected by a pulsed electrochemical detector in the pulsed amperometric detection mode. The quantification was carried out by use of arabinose and xylose as external standards at defined concentrations. The data were collected and analyzed on computers equipped with Chromeleon 6.80 Sp2 Build 1472 software (Dionex Corp., Sunnyvale, CA, USA).

## 2.6. Enzyme activity assays

β-Xylosidase and arabinofuranosidases activities were assayed by the hydrolysis of 2.5 mM *p*-nitrophenyl xyloside (pNPX) or *p*-nitrophenyl arabinoside (pNPA), respectively, in 125 mM sodium acetate buffer pH 5 at 40°C for 10 min. In each case the enzymes were added in amounts sufficient to promote an absorbance increase between 0.1 and 1.5 at 410 nm. The

reaction was terminated by addition of 1 M Na<sub>2</sub>CO<sub>3</sub>. One unit, U, of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol · min<sup>-1</sup> of pNPX or pNPA. The molar specific activity is reported as U · (μmol enzyme)<sup>-1</sup>.

### 3. Results and discussion

#### 3.1. Substrate concentration

The substrate for β-xylosidase is in fact the β-1,4-bond of the terminal D-xylopyranosyl at the non-reducing end of the xylose-backbone of arabino-xylooligosaccharides, and thus not the full arabinoxylan molecule. The “substrate concentration” of this β-1,4-bond can be calculated from the number of non-reducing ends. With this particular WEAX, the estimated molar weight was 270 kDa, and the purity was 95% (Table 1). Assuming a narrow, uniform distribution of molecular weights (degree of polymerization), the initial average molar concentration of the non-reducing ends,  $C_{nre}$ , for 2 g · L<sup>-1</sup> of the arabinoxylan could then be roughly predicted to be:

$$C_{nre} = \frac{0.95 \cdot 2.0 \frac{\text{g}}{\text{L}}}{270 \frac{\text{kg}}{\text{mole}}} = 0.0070 \text{ mM}$$

However, the β-xylosidase derived from *T. reesei* can only catalyze the hydrolysis of the terminal, non-reducing D-xylopyranosyl residue if both of the two terminal D-xylopyranosyl residues are unsubstituted [9]. Previous data for WEAX with an A:X ratio of approximately 0.6 have shown that the distribution of singly vs. doubly substituted arabinoses is approximately ~1/3 vs. ~2/3 [17, 18]. This distribution means that approximately 40% of the xylopyranosyl residues in the xylan backbone in WEAX are substituted with α-L-arabinofuranosyl (Table 1). Accordingly, about 60 % of the xylose residues in WEAX are unsubstituted (Table 1). If the arabinose substitutions are randomly distributed, the probability ( $P$ ) that the first xylose, counted from the non-reducing end, is un-substituted is thus 60 %. In turn, the probability – by a rough estimate – that the first two xyloses, counted from the non-

reducing end, are un-substituted is  $0.60 \cdot 0.60 = 0.36$  (36 %). The estimated real initial substrate concentration for  $\beta$ -xylosidase,  $[S_0]_{\beta\text{-xylosidase nre}}$ , was therefore only 36 % of the  $C_{nre}$ , namely  $\sim 0.0025$  mM at an arabinoxylan concentration of  $2 \text{ g}\cdot\text{L}^{-1}$  (Table 1). In reality, the substrate concentration for successful hydrolysis by  $\beta$ -xylosidase may be somewhat less, considering that  $\beta$ -xylosidase preferably attacks relatively short oligomers, rather than longer arabinoxylan molecules.

### 3.2. Hydrolysis of *p*-nitrophenyl substrates by $\beta$ -xylosidase and arabinofuranosidases

The enzyme reaction rate data (Table 3) confirmed that the  $\beta$ -xylosidase had high activity on the pNPX, but also indicated that the  $\beta$ -xylosidase exerted activity on pNPA and thus apparently exhibited arabinofuranosidase activity. The assay results thus suggested that the  $\beta$ -xylosidase from *T. reesei* is able to accommodate  $\alpha$ -L-arabinofuranose in its active site suggesting an active site structure similar to the AF<sub>An</sub>. Since  $\alpha$ -L-Arabinofuranose and  $\beta$ -D-xylopyranose have identical configurations in positions C1 and C4 [19], both pentoses may therefore fit into the binding pocket of the  $\beta$ -xylosidase enzyme. In analogy to what has been observed for a “multifunctional” arabinofuranosidase [19], the difference in activity of the  $\beta$ -xylosidase on the two assay substrates could, therefore, be a result of the enzyme’s ability to accommodate the  $\alpha$ -L-arabinofuranose in the active site, but inability to foster the distortion required for efficient catalysis of furanose structures. The activity of  $\beta$ -xylosidase towards both substrates (pNPA and pNPX) is therefore not necessarily the result of dual specificity, but rather a consequence of imprecise substrate recognition by the enzyme on these artificial substrates. The results obtained using pNPA and pNPX substrates may moreover not necessarily reflect the  $\beta$ -xylosidase specificity on genuine arabinoxylans [3].

### 3.3 Hydrolysis of WEAX by $\beta$ -xylosidase and arabinofuranosidases

#### 3.3.1. Arabinose release from WEAX

As expected, the AF<sub>An</sub> exhibited activity on the pNPA, while the AF<sub>Ba</sub> did not which is in accordance with previous reported activities for AF<sub>Ba</sub> (Table 3). In contrast, however, using the genuine arabinoxylan substrate, the AF<sub>Ba</sub> catalyzed the release of approximately three fold higher amounts of arabinose, and at a ~5 fold higher initial rate, than AF<sub>An</sub> when added at equimolar concentrations (Figure 1). The “substrate” for both of these two arabinofuranosidases is the (1→3) arabinofuranosyl bond, but attached to singly (AF<sub>An</sub>) and doubly (AF<sub>Ba</sub>) substituted xylose, respectively. Since the distribution of singly (1→3) arabinofuranosyl bonds vs. doubly (1→3) arabinofuranosyl bonds were 1:1 (Table 1), the “substrate concentration” for the AF<sub>Ba</sub> was in fact the same of that of the AF<sub>An</sub>. The results therefore indicate a higher pseudo first order rate constant per mole of enzyme (corresponding to  $k_{cat}/K_M$  in Michaelis-Menten enzyme kinetics) of the AF<sub>Ba</sub> enzyme as compared to that of the AF<sub>An</sub> activity on WEAX under the employed reaction conditions. The addition of  $\beta$ -xylosidase alone did not produce any arabinose release (Figure 1). Neither did equimolar additions of  $\beta$ -xylosidase together with any of the two arabinofuranosidases have a significant influence on the arabinose release as compared to the releases obtained with the arabinofuranosidases individually. When the molar concentration of the  $\beta$ -xylosidase was doubled, the arabinose releases even decreased for both of the arabinofuranosidase +  $\beta$ -xylosidase combinations (Figure 1). As discussed above, the  $\beta$ -xylosidase can be expected to also bind arabinofuranose in its active site, but without catalyzing the release of arabinose on the genuine arabinoxylan substrate. The phenomenon that additional  $\beta$ -xylosidase decreased the arabinose release can be explained by  $\beta$ -xylosidase occupying the same locations on the substrate as arabinofuranosidase. In this way it can partially, sterically hinder the arabinofuranosidase catalyzed removal of arabinose. This type of inhibition has been observed previously for multi-enzymatic hydrolysis of pectinaceous substrates and has been called “competitive, non-productive enzyme adsorption” [20, 21].

### 3.3.2. Xylose release from WEAX

Despite the higher release of arabinose by the action of the  $AF_{Ba}$ , a higher xylose release was produced by the combination of  $AF_{An}$  +  $\beta$ -xylosidase than by  $AF_{Ba}$  +  $\beta$ -xylosidase (Figure 2). That the xylose release was improved with addition of the arabinofuranosidase as compared to the release catalyzed by  $\beta$ -xylosidase alone proved a synergistic effect by these enzymes on the xylose release. As expected, the  $AF_{An}$  exhibited a stronger synergistic effect with  $\beta$ -xylosidase than  $AF_{Ba}$  on xylose release. A mechanism explaining the differences in the synergistic effects is suggested in Figure 3:  $AF_{An}$  only attacks the (1→3) bonds of singly substituted arabinose and hence directly provides unsubstituted D-xylopyranosyls. In turn this enhances the probability of more unsubstituted xyloses at (or near) the non-reducing ends for  $\beta$ -xylosidase to attack.  $AF_{Ba}$  attacks the 1→3 linked arabinose on doubly substituted xyloses, and catalyze the release of the 1→3 linked arabinose. After removing the 1→3 linked arabinose, the  $\beta$ -xylosidase still cannot work on the xylan backbone, because the  $\alpha$ -1→2 linked arabinose blocks the binding site. The observation that the treatment with the combination  $AF_{Ba}$  +  $\beta$ -xylosidase gave higher xylose release than  $\beta$ -xylosidase treatment alone (Figure 2) may indicate, however, that the  $AF_{Ba}$  activity was partly able to also attack either the remaining  $\alpha$ 1→2 linked arabinose on originally doubly substituted xylose or – less likely [11, 14] – also acted on the arabinose that was 1→3 linked to singly substituted xylose. In any case, the levels of xylose yield were low by all enzyme combinations as compared to the arabinose release. For the  $\beta$ -xylosidase alone, the maximal xylose release, after 4 hours was only approximately  $0.003 \text{ g} \cdot \text{L}^{-1}$  (Figure 2). This xylose concentration is equivalent to approximately 0.02 mM. This corresponds to the achievement of ~7 subsequent cuts per molecule *initially* having accessible xyloses in their non-reducing ends (initially:  $[S_0]_{\beta\text{-xylosidase}}$ ), which is equivalent to a "degree of hydrolysis" of these accessible arabinoxylans of  $7/1277 \approx 0.55 \%$ . However, out of all the xyloses bound in the arabinoxylan backbone, which



was ~9 mM at 2.0 g · L<sup>-1</sup> for this substrate, the degree of hydrolysis was much less: 0.02/9 ≈ 0.22 %. The reaction curve for the β-xylosidase treatment alone also tended to run close to “substrate depletion”. The question then becomes how close this degree of hydrolysis was to the true theoretically maximal release accomplished by β-xylosidase considering the arabinosyl-substitutions. In continuation of the derivation of [S<sub>0</sub>]<sub>β-xylosidase nre</sub>, the theoretically maximal xylose release from the soluble wheat arabinoxylan substrate by β-xylosidase alone, xylose<sub>max β-xyl</sub> can be calculated by first considering that (without any arabinofuranosidase action) only 60% of the xylopyranosyls in each arabinoxylan backbone are unsubstituted, only 766 mole of xylose per each mole of the wheat arabinoxylan can be released, 0.6 x 1277 = 766. The maximal xylose concentration obtainable via β-xylosidase action alone therefore becomes:

$$C_{\text{xylosemax}\beta\text{xyl}} = \sum_{n=2}^{n=766} P_n C_{\text{nre}} = \left( P_2^2 + P_3^3 \dots + P_{766}^{766} \right) C_{\text{nre}}$$

Where  $P_n = \frac{766-n}{1277-n}$  and  $n$  represent the xylosidyl residue number counting from the non-reducing end. 1277 is the estimated total number of xylosidyl residues of the arabinoxylan backbone and (1277 x 60%)/100% = 766 is the estimated total number of non-substituted xylosidyl residues. Xylosidyl residues beyond xylosyl-residue number 20 counting from the non-reducing contributes only little to  $C_{\text{xylosemax}\beta\text{xyl}}$ .  $P$  can therefore be considered constant and the formula can be reduced to:

$$C_{\text{xylosemax}\beta\text{xyl}} = \sum_{n=2}^{n=766} P^n C_{\text{nre}} = \left( P^2 + P^3 \dots + P^{766} \right) C_{\text{nre}}$$

The geometric series may further be reduced to

$$C_{\text{xylose max } \beta\text{-xyl}} = \left( \frac{1}{1-P} - P - 1 \right) C_{\text{nre}}$$

When considering that the true xylose<sub>max β-xyl</sub> in this case becomes  $\sim 0.89 \cdot C_{\text{nre}} = 0.006 \text{ mM}$  the release obtained during the 4 hours of reaction (Figure 2) was in fact about 4 times larger than the xylose<sub>max β-xyl</sub>. This realization must reflect that some of the assumptions for the theoretical calculations are not valid. The estimation of the DP is obviously somewhat “overprecise”, and may in fact cover a range of DP-profiles, in the present case presumably relatively more lower DP molecules than higher DP arabinoxylan molecules. Another main issue is that the arabinosyl-substitutions may in fact not be evenly distributed on the xylan backbone, which is in complete accord with the available knowledge [6, 7]. Clearly, the inclusion of the distributional variability of the arabinosyl-substitutions demands more detained modeling of the substrate.

The highest increase in xylose release to  $0.0072 \text{ g} \cdot \text{L}^{-1}$  after 4 hours by the treatment with the combination  $\text{AF}_{\text{An}} + \beta\text{-xylosidase}$  represented the catalytic hydrolysis of almost 7 bonds in the xylan backbone of each of the arabinoxylan molecules present (because the action of  $\text{AF}_{\text{An}}$  will increase the  $[\text{S}]_{\beta\text{-xylosidase nre}}$  and increased the degree of hydrolysis of the xylan to  $\sim 0.5\%$  (considering the total amount of xyloses available).

As also seen for the arabinose release, the doubling of the  $\beta\text{-xylosidase}$  molar dosage also lowered the xylose release. This suggests the same competitive, non-productive enzyme adsorption leading to steric blocking of the arabinofuranosidase attacks, discussed above, which in turn then affected the provision of more accessible substrate sites, i.e. unsubstituted xyloses, for  $\beta\text{-xylosidase}$  attack.

### 3.4. Hydrolysis of WEAX by xylanase, $\beta\text{-xylosidase}$ and arabinofuranosidases.

#### 3.4.1. Arabinose release from WEAX

1 Addition of xylanase and  $\beta$ -xylosidase each helped increase the arabinose release to different  
2 extents (Figure 4). However, neither xylanase nor  $\beta$ -xylosidase alone catalyzed the release of  
3 arabinose without the presence of arabinofuranosidases.

#### 4 5 3.4.2. Xylose release from WEAX

6 When comparing the xylose release obtained from the combined treatments with xylanase +  
7  $\beta$ -xylosidase + AF<sub>An</sub> and  $\beta$ -xylosidase + AF<sub>An</sub>, respectively, the addition of xylanase  
8 dramatically increased the xylose release by ~23 times during 30 minutes of reaction, while  
9 the difference between xylanase +  $\beta$ -xylosidase + AF<sub>An</sub> and  $\beta$ -xylosidase treatment alone was  
10 ~25 times (Figures 2 and 5). Analogously, the treatment combination xylanase +  $\beta$ -  
11 xylosidase increased the xylose release rate by ~25 times as compared to the result of  $\beta$ -  
12 xylosidase treatment alone during the first 30 minutes of reaction. Treatment with the total  
13 combination of xylanase +  $\beta$ -xylosidase + AF<sub>An</sub> + AF<sub>Ba</sub> did not result in a significantly higher  
14 xylose release rate than the xylanase +  $\beta$ -xylosidase + AF<sub>An</sub> treatment, which confirmed that  
15 despite a higher arabinose release (Figure 4) the AF<sub>Ba</sub> treatment did not produce additional  
16 accessible substrate sites for neither xylanase nor  $\beta$ -xylosidase (Figure 3).

17 The data indicated that the breaking down of the xylan backbone to smaller oligosaccharides,  
18 and thus exposing more (unsubstituted) non-reducing ends for  $\beta$ -xylosidase, is more  
19 important than removing the substituted arabinose in order to release more xylose from  
20 arabinoxylan.

21 Quantitatively, assuming a linear increase in accessible non-reducing ends for  $\beta$ -xylosidase  
22 during the first 30 minutes of xylanase action, the 25 fold increase indicates that the xylanase  
23 action may have increased the available, unsubstituted xyloses in the non-reducing ends from  
24 initially approximately 0.0025 mM to approximately 0.07 mM. The maximal levels of xylose  
25 released, of 0.11 g · L<sup>-1</sup> (Figure 5) represented a degree of hydrolysis (to liberate xylose) of ~8

% of the xylan in the substrate, equivalent to about 104 cuts in the xylan backbone of each arabinoxylan molecule present.

Since the different enzymes were added at equimolar levels, the results indicate that xylanase was the rate-limiting activity in the context. The combination xylanase + AF<sub>An</sub> gave very low xylose release which confirmed that the xylanase employed had no  $\beta$ -xylosidase side activity.

### 3.5 Initial rate study with different substrate concentrations

Based on the arabinose and xylose release data (Figures 1,2, 4 and 5) the initial 30 minutes of reaction were taken as the initial rate period. The measurement of the kinetics for the  $\beta$ -xylosidase catalyzed depolymerization of WEAX is particularly difficult since a balance has to be made between obtaining sufficient xylose for the initial quantification and employing high substrate concentrations since the substrate is viscous at high substrate concentrations. Surprisingly, both the xylose and the arabinose release rates ( $v$ ) measured at 30 minutes for different, selected enzyme combinations in response to the increase in substrate concentration ( $[S]$ ) appeared to follow the classic rectangular hyperbola Michaelis-Menten type curves: ( $a - v)([S]+b) = \text{constant}$  (For Michaelis-Menten kinetics  $a$  is a constant equivalent to  $V_{max}$  and  $b$  is a constant equivalent to  $K_M$ ). Similar to Michaelis-Menten curves, the xylose and arabinose release rates showed an initial linear increase in reaction rate with an increase in  $[S_0]$  until the  $V_{max}$  is eventually reached (Figures 6 and 7). For the release of xylose from the  $\beta$ -xylosidase activity the rate response was linear, increasing with the substrate concentration, while it showed saturation above  $\sim 2.5 \text{ g}\cdot\text{L}^{-1}$  arabinoxylan for the synergistic reactions with xylanase (Figure 6). Although the introduction of xylanase and the AF<sub>An</sub> functions to *increase* the true  $[S_0]$  for the  $\beta$ -xylosidase, i.e.  $[S_0]_{\beta\text{-xylosidase nre}}$ , which fundamentally hinders the measurement of initial rates and Michaelis-Menten kinetic parameters such as  $K_M$  and  $V_{max}$ , apparent could be derived by non-linear regression with reasonable confidence intervals (data not shown).

The apparent  $V_{max}$  and  $K_M$  values for the combinations xylanase +  $\beta$ -xylosidase + AF<sub>An</sub> and

xylanase +  $\beta$ -xylosidase were quite similar and had equal  $V_{max} \approx 0.05 \text{ mmol} \cdot (\text{min} \cdot \text{L})^{-1}$ ; resulting in a  $k_{cat}$  of  $\sim 20000 \text{ s}^{-1}$  ( $K_M \approx 8 \text{ g} \cdot \text{L}^{-1}$  arabinoxylan). The similarity of the values confirmed that the synergistic effect of the xylanase on the  $\beta$ -xylosidase activity by far exceeded that of the AF<sub>An</sub>. In other words, the provision of more (unsubstituted) non-reducing ends resulting from xylanase action was more efficient to boost the  $\beta$ -xylosidase activity than provision of more (randomly) unsubstituted xylosidyl residues in the arabinoxylan backbone. In comparison the similarly derived kinetic parameters for  $\beta$ -xylosidase action alone was  $V_{max} \approx 0.002 \text{ mmol} \cdot (\text{min} \cdot \text{L})^{-1}$ ;  $K_M \approx 10 \text{ g} \cdot \text{L}^{-1}$  arabinoxylan. The xylanase activity was thus rate-limiting for the xylose release and the xylanase activity increased the xylose release so much that the synergistic effect from AF<sub>An</sub> was in effect abolished.

In any case, the overall substrate concentration for  $\beta$ -xylosidase was not constant, but increased during the reaction, and thus exceeded the initial substrate concentration by several folds. For  $2 \text{ g} \cdot \text{L}^{-1}$  arabinoxylan, the initial rate difference between xylanase +  $\beta$ -xylosidase + AF<sub>An</sub> and  $\beta$ -xylosidase alone was  $\sim 25$  times (Figure 6). Add to this, that the activity of the  $\beta$ -xylosidase is presumably higher on short-chain oligomers than on longer arabinoxylan molecules. Since the DP of the products of the xylanase activity kept changing during the reaction, the new  $[S_0]_{\beta\text{-xylosidase nre}}$  will be dynamic and cannot be predicted by simple models. However, this true substrate concentration for  $\beta$ -xylosidase remains proportional to the arabinoxylan concentration. The higher the arabinoxylan concentration, the higher the substrate concentration,  $[S_0]_{\beta\text{-xylosidase nre}}$ , potentially available for  $\beta$ -xylosidase. Therefore, we used the crude arabinoxylan concentration as the “substrate” concentration in Figure 6 and Figure 7.

The initial rate (30 minutes) of arabinose release also appeared to follow the Michaelis-Menten kinetics (Figure 7). The apparent  $V_{max}$  and  $K_m$  of the AF<sub>An</sub> when acting together with

the xylanase and  $\beta$ -xylosidase, derived from the data in Figure 8, were  $V_{max} \approx 0.004 \text{ mmol} \cdot (\text{min} \cdot \text{L})^{-1}$ ;  $K_M \approx 8 \text{ g} \cdot \text{L}^{-1}$  arabinoxylan.

#### 4. Conclusions

The data obtained demonstrated that the xylanase activity is the dominating rate-limiting enzyme activity for xylose release during enzymatic hydrolysis of WEAX by the cooperative action of arabinofuranosidases having different specificities, together with xylanase and  $\beta$ -xylosidase. Xylanase exhibited a significant synergistic effect on xylose release when acting together with  $\beta$ -xylosidase. Comparably, the arabinofuranosidases, including the AF<sub>An</sub> releasing (1 $\rightarrow$ 3) bonded arabinofuranosyls from singly substituted xyloses, exerted lower synergistic effects with  $\beta$ -xylosidase on xylose release. The data confirmed the different actions and the different arabinoxylan hydrolysis consequences of the two arabinofuranosidases: Although the AF<sub>Ba</sub> had higher specific molar catalytic activity on the arabinoxylan, the AF<sub>An</sub> exhibited better synergy with  $\beta$ -xylosidase to release xylose. Doubling of the  $\beta$ -xylosidase enzyme dosage did not increase either xylose or arabinose release, rather did the surplus of  $\beta$ -xylosidase decrease the yields of both arabinose and xylose, presumably as a result of competitive non-productive binding of the surplus  $\beta$ -xylosidase to the substrate, which blocked the access of the other enzymes.

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2

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## FIGURE LEGENDS

**Figure 1.** Arabinose release from the hydrolysis by  $\beta$ -xylosidase and arabinofuranosidases. 2

\*  $\beta$ -xylosidase indicates that the enzyme dosage of  $\beta$ -xylosidase was doubled.

**Figure 2.** Xylose release from the hydrolysis by  $\beta$ -xylosidase and arabinofuranosidases.

**Figure 3.** Hypothesis for the synergistic effect of different types of arabinofuranosidase activity on xylose release.

**Figure 4.** Arabinose release from the hydrolysis with the addition of xylanase.

**Figure 5.** Xylose release from the hydrolysis with the addition of xylanase.

**Figure 6.** Reaction rates (xylose) on different substrate concentrations.

**Figure 7.** Reaction rates (arabinose) on different substrate concentrations.

1 **Table 1.** Water-soluble wheat arabinoxylan (WEAX) substrate details

MW (kDa)	A:X ratio	Purity	Substituted xylose 40%			DP <sup>2</sup>	[S <sub>0</sub> ] <sub>nre</sub> for β- xylosidase attack <sup>3</sup>
			Singly substituted xylose	Doubly substituted xylose	Unsubstituted xylose		
270	0.6	95%	~50% <sup>1</sup>	~50% <sup>1</sup>	60%	~1277	0.0025 mM ≈ 36 % of the initial C <sub>nre</sub> at 2 · g L <sup>-1</sup> of arabinoxylan

2 <sup>1</sup> From reference [17];

3 <sup>2</sup> DP: Average degree of polymerization of the xylan backbone: Assuming an equal distribution of the molecular  
4 size of the arabinoxylan, the average number of arabinose + xylose in each molecule can be calculated to:  
5  $270000 = 150.14n - 18.02(n-1) \Rightarrow n = 2043$ . At A:X = 0.6  $\Rightarrow$  X = 1277.

6 <sup>3</sup> Assuming an narrow DP distribution of the xylan backbone, that the arabinosyl substitutions are distributed  
7 evenly across the xylan backbone, and that all the arabinoxylan molecules are all attacked equally by the β-  
8 xylosidase.

9

1    **Table 2.** Enzymes used for the enzymatic hydrolysis of WEAX

Enzyme	Microorganism	EC	Family	pH optimum	Temp. optimum	Ref.
$\alpha$ -L-arabinofuranosidase	<i>Aspergillus niger</i>	EC.3.2.1.55	GH 51	4	40	[18]
$\alpha$ -L-arabinofuranosidase	<i>Bifidobacterium adolescentis</i>	EC.3.2.1.55	GH 43	6	40	[12]
$\beta$ -xylosidase	<i>Trichoderma reesei</i>	EC.3.2.1.37	GH 3	5	50	[ 3]
Endo-1,4- $\beta$ -xylanase (BsX <sub>mut</sub> )	<i>Bacillus subtilis</i>	EC.3.2.1.8	GH 11	6	50	[16]

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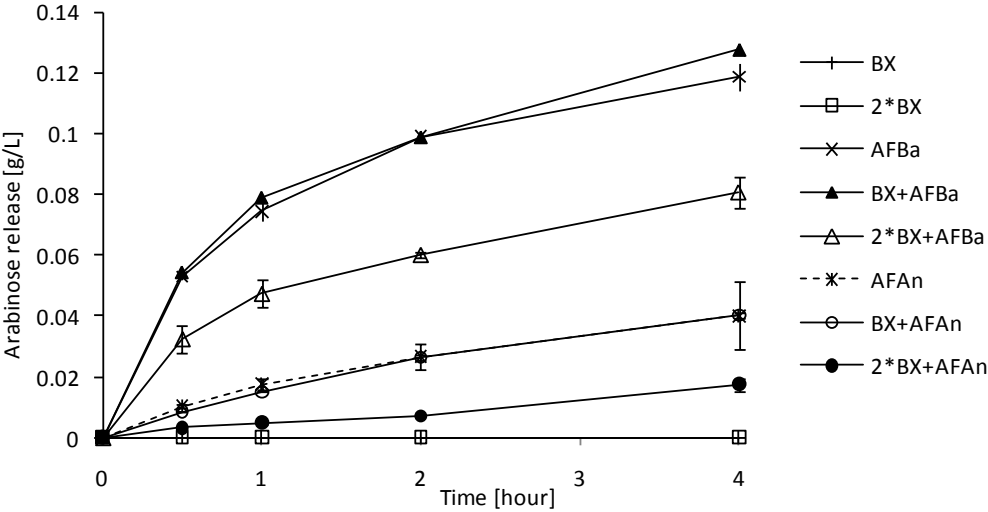
1   **Table 3** Activity data by *p*-nitrophenol xyloside (pNPX) and *p*-nitrophenol arabinoside assays  
 2   for enzyme activity

Substrate	Enzyme activity (U · μmole enzyme)		
	β-xylosidase	AF <sub>An</sub>	AF <sub>Ba</sub>
pNPX	2091	0	0
pNPA	60.5	647	0

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1    Figure 1

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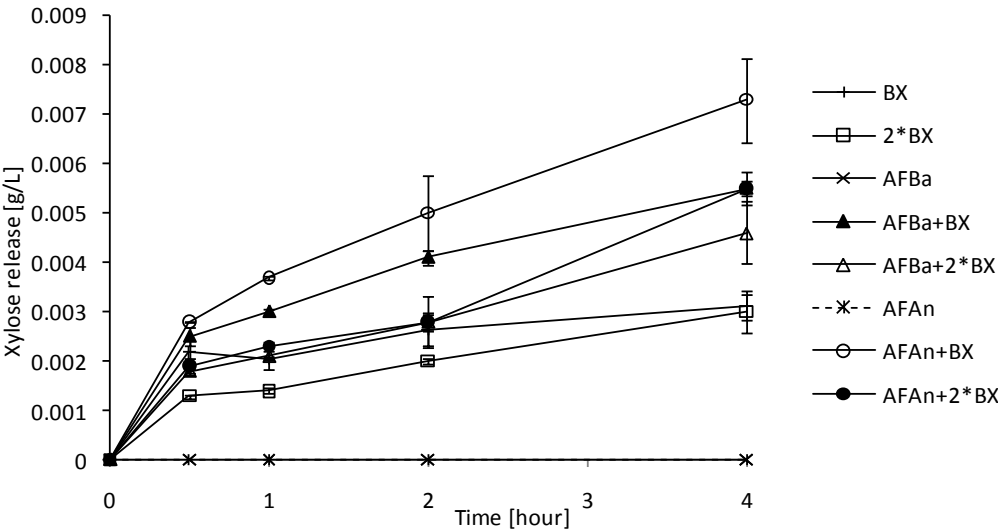


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1    Figure 2

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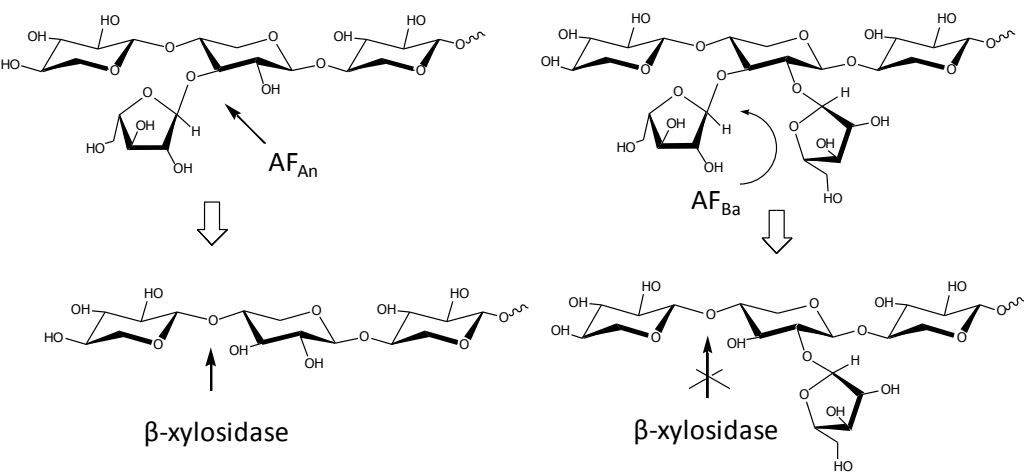
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1    Figure 3

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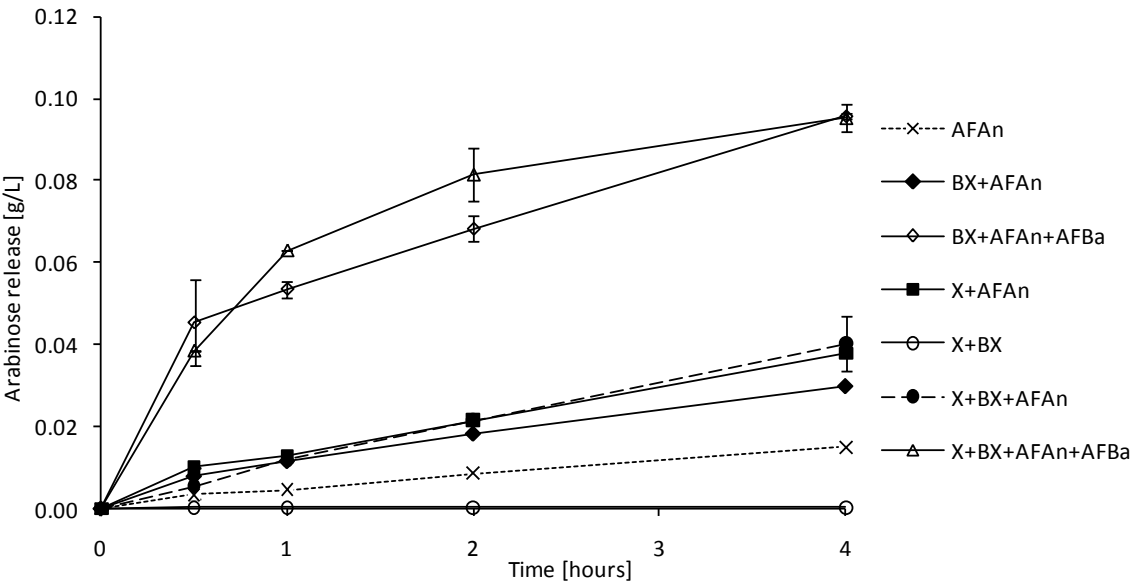
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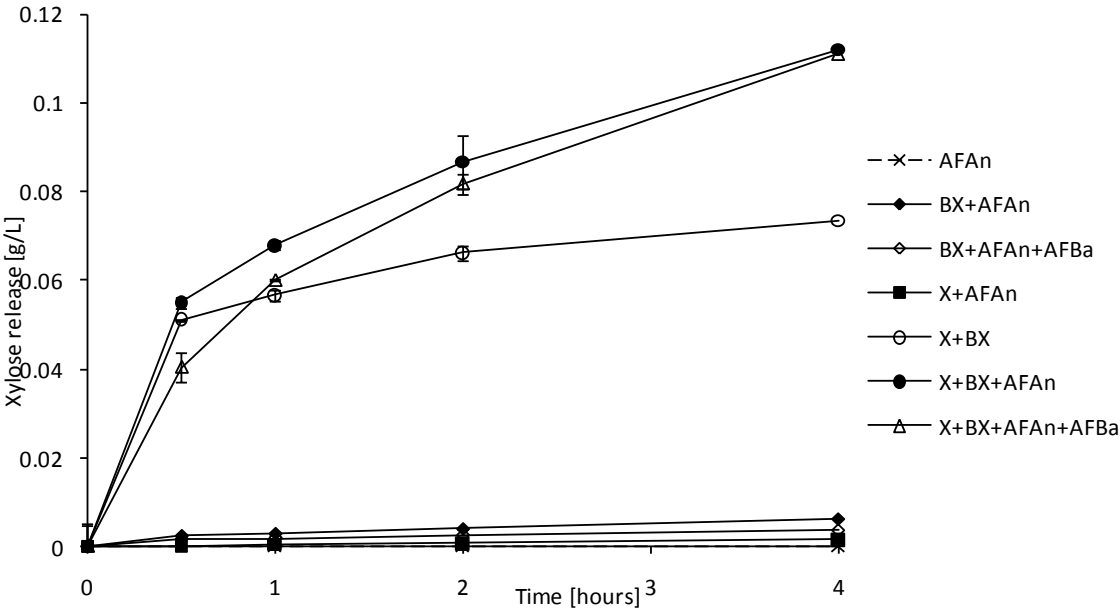
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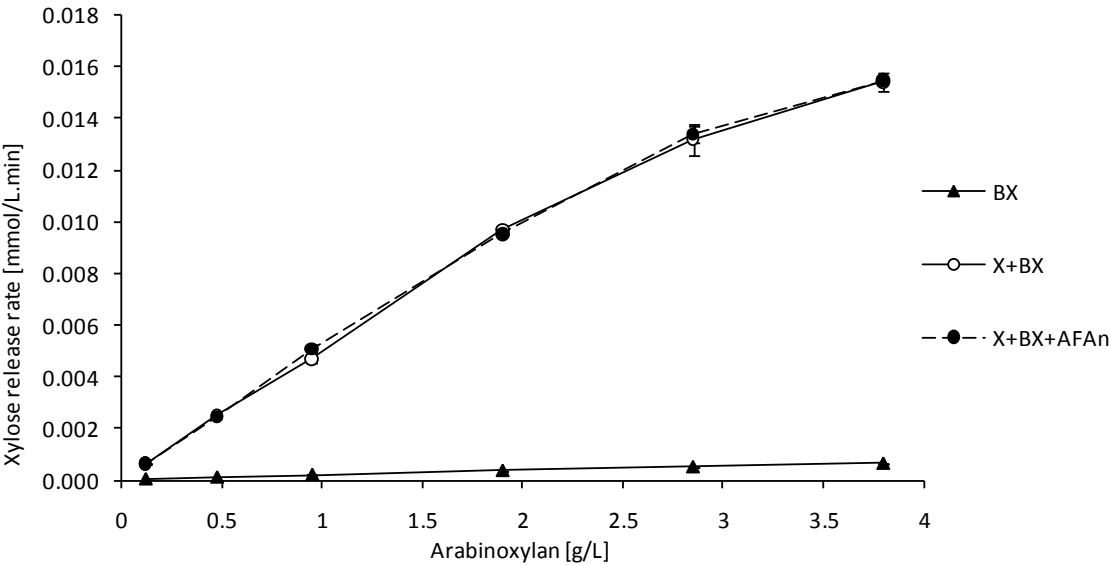
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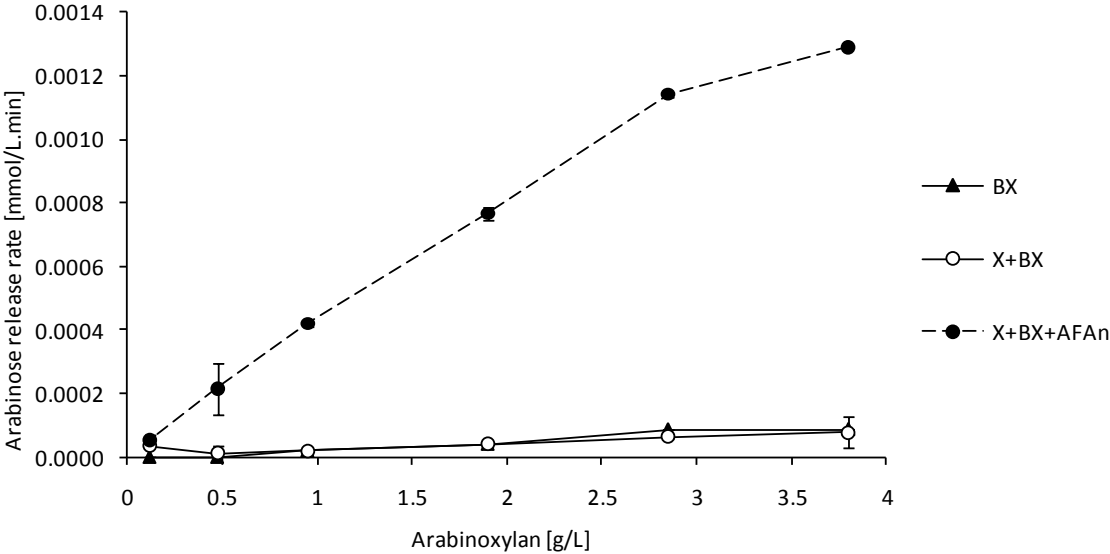
1    Figure 6



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1    Figure 7

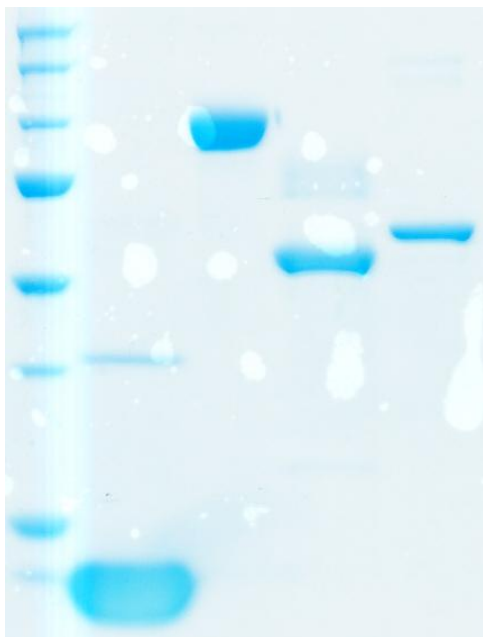


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1 Supplement 1

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5 **Supplement 1.** SDS PAGE of the enzyme preparations used in this study.

6 Lane 1: Molecular weight marker; Lane 2: Xylanase (20 kDa); Lane 3: β-Xylosidase (87 kDa);

7 Lane 4: AF<sub>Ba</sub> (59 kDa); Lane 5: AF<sub>An</sub> (62 kDa).

8

## Chapter 3 Kinetics and substrate selectivity of the TAXI resistant *Bacillus subtilis* xylanase

This chapter is extended in the form of Paper 2:

Kinetics and substrate selectivity of a *Triticum aestivum* xylanase inhibitor (TAXI) resistant D11F variant of *Bacillus subtilis* XynA xylanase

Rasmussen L.E., Sørensen J.F., Meyer A.S.

Journal of Biotechnology, 146: 207-214, 2010

### 3.1 Key points

The purpose of this article was to examine the kinetics and substrate selectivity of BsX<sub>mut</sub> as opposed to BsX. The understanding of the changes in the catalytic capabilities of the engineered enzymes is important for predicting the enzyme catalyzed outcome in genuine applications. The evaluation was done by assessing the variant and wildtype xylanase on xylan containing substrates, which differed in solubility and structure. Commercial WE-AX and WU-AX, both extracted from wheat, together with birchwood xylan were assessed. The hydrolysis of these relatively pure substrates was compared with wheat bran, which is a more complex substrate containing other polymers besides arabinoxylan. The detailed structure of each substrate is described in the following section.

#### 3.1.1 Origin and main components of the applied substrates

The assessed WE-AX from wheat endosperm has an average ratio of arabinose to xylose (A/X) of 0.6 which for arabinoxylan generally represents the degree of substitution. Previous data for WEAX with an A:X ratio of approximately 0.6 have shown, that the distribution of singly vs. doubly substituted arabinoses is approximately ~1/3 vs. ~2/3 (Sørensen et al., 2007). This distribution means that approximately 40% of the xylopyranosyl residues in the xylan backbone in WEAX are substituted with arabinofuranosyl. Accordingly, about 60 % of the xylose residues in WEAX are un-substituted. The structure of WU-AX is very close to that of WE-AX (Izydorczyk and Biliaderis, 1995). The difference in extractability is accompanied by varying physio-chemical properties, where WE-AX lead to highly viscous solution when brought in aqueous media and WU-AX on the other hand, have strong water holding capacity (Courtin and Delcour, 2001).

Wheat bran, the non-starchy outer part of the wheat grain, is a by-product from the milling of white flour. It consists mainly of arabinoxylan (22-25% (w/w)), cellulose (7-11% (w/w)) and  $\beta$ -glucans. Also, rather high amounts of starch are present in the bran (11-30% (w/w)), besides protein (14-17% (w/w)) and lignin (3-10% (w/w)). In general, arabinoxylan purified from wheat

bran is mainly substituted with arabinose residues at the C(O)-2 and/or the C(O)-3 positions, while some side-chains of (4-O-methyl)- $\alpha$ -D-glucuronic acids are present as well (Brillouet et al., 1982). However, the A/X varies for arabinoxylan derived from different parts of the bran (Figure 7). In endosperm, an A/X ratio of 0.6 is usually found, but large natural variations are observed. Arabinoxylan originating from the aleurone layer and from the inner pericarp, testa and nucella tissue are characterized by lower A/X ratio (0.3-0.4), whereas arabinoxylan in the outer pericarp have a considerable higher degree of substitution (A/X  $\sim$  1) (Ordaz-Ortiz et al., 2004).

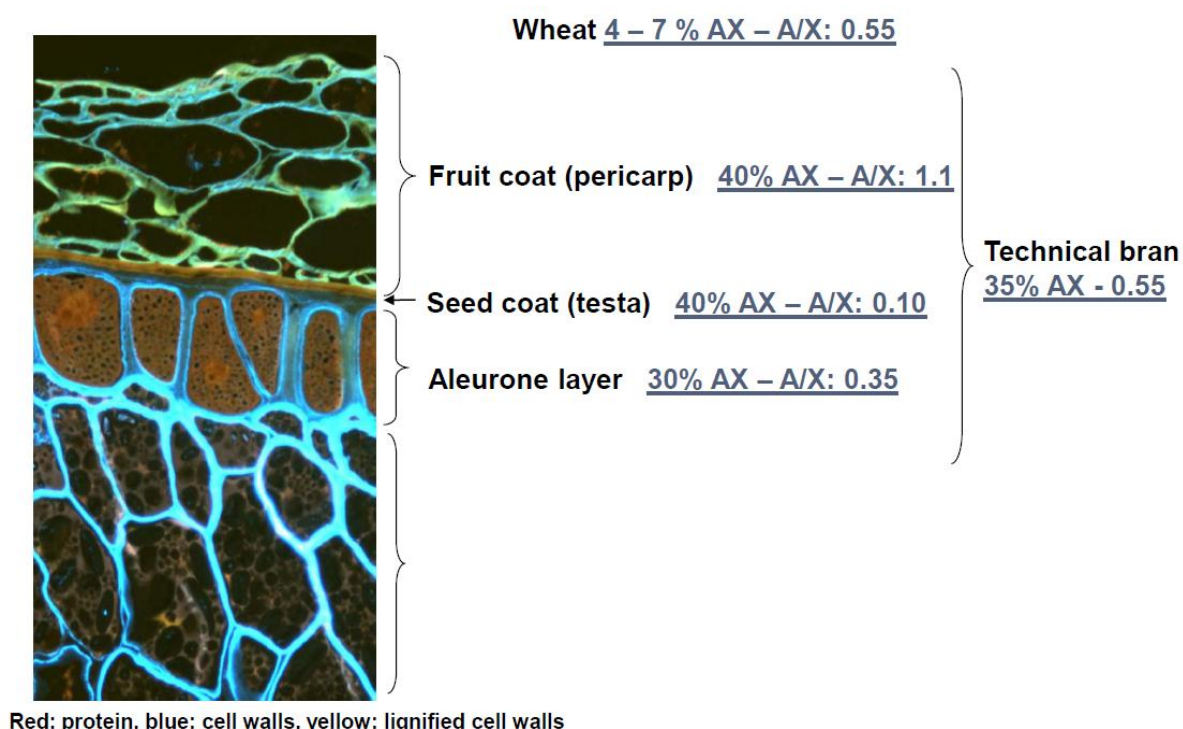


Figure 7. Arabinoxylan distribution and degree of substitution in the wheat grain (Courtin 2010)

Contrary to the arabinoxylan present in WE-AX, WU-AX and wheat bran, birchwood xylan is a typical hardwood xylan with a  $\beta$ -1,4-xylan backbone practically free of arabinofuranosyl groups. A commercial birchwood xylan were used and the manufacturer claimed that the xylose content was more than 90%.

The structural models of xylan extracted from various sources are presented in Figure 8.



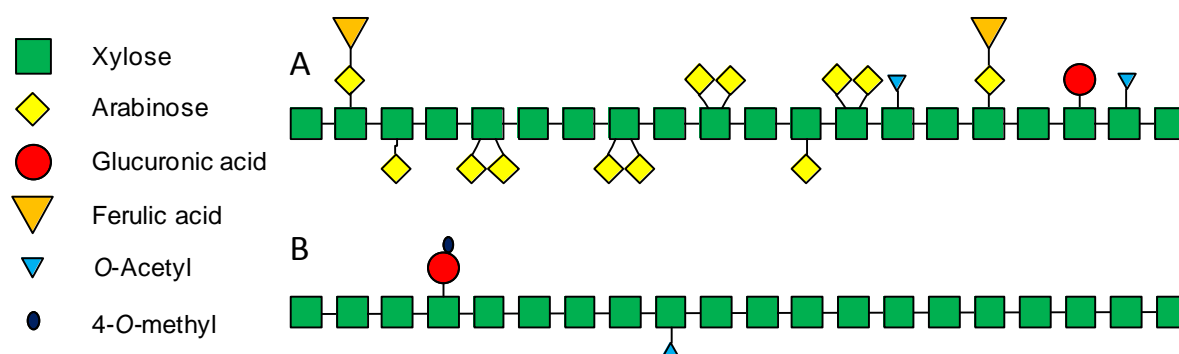


Figure 8. Compilation of structural models for xylan. A: Arabinoxylan (WE-AX/WU-AX/wheat bran); B: Glucuronoxylan (Birchwood xylan). The xylopyranosyl residues in the xylan backbone are  $\beta$ -1,4 linked, whereas the arabinofuranosyl and glucopyranosyl uronic acid substituents are  $\alpha$ -1,2- and/or  $\alpha$ -1,3-linked. Ferulic acids are estrified to the C(O)-5 of the arabinofuranosyl units, whereas acetyl substitutions are estrified to the xylopyranosyl residues in the xylan backbone. In dicots specially, the glucopyranosyl uronic acid is methylated.

### 3.1.2 Xylanase substrate selectivity

As described previously, the impact of xylanases in bread making strongly depends on their sensitivity towards xylanase inhibitors. However, the xylanases relative activity towards WU-AX and WE-AX substrates, referred to as xylanase substrate selectivity (Moers et al., 2005) also impacts in the enzyme functionality in cereal-based biotechnological processes (Berrin and Juge 2008). The concept of substrate selectivity is rather new and its biochemical basis remains as yet unknown. Xylanases within the same GH family and thus with similar specificity, differ substantially in substrate selectivity. Thus, classification of GH family, alone, is not sufficient to predict the preference for WEAX versus WUAX (Bonnin et al., 2006; Moers et al., 2005). For instance, previous results have shown that the GH11 *B. subtilis* xylanase, which preferentially hydrolyses WU-AX and leaves WE-AX, beneficially affects bread loaf volume (Courtin and Delcour, 2001; Courtin et al., 2001). On the other hand, the GH10 xylanase from *Aspergillus aculeatus*, which preferentially degrades WE-AX, is used to improve gluten agglomeration in industrial wheat gluten separation (Christophersen et al., 1997; Frederix et al., 2003).

## 3.2 Conclusion

This study established that the overall activity of the BsX<sub>mut</sub> was significantly reduced on different polymeric xylan substrates. Based on the structure analysis, the reduced activity of BsX<sub>mut</sub> was proposed to be due to the steric hindrance caused by the D11F mutation. This confirms the hypothesis that the double mutation D11F/R122D had an effect on the kinetics of BsX<sub>mut</sub> as opposed to BsX. Furthermore, it was concluded that the catalytic effects and rates of both xylanases on substrates, that differ in complexity, may be related to the general structure of the active site for GH11 xylanases. That is, the cleft-shaped active site resulted in a restricted catalytic behavior, where substituents presented a hindrance. Thus, also the

hypothesis about a correlation between the extent of substrate substitution and the enzymatic solubilization of xylan could be confirmed. In Paper 2, a more in depth characterization of the xylanase substrate selectivity was wanted and therefore a distinction was made between the initial and the end point selectivity. The overall selectivity factors of BsX and BsX<sub>mut</sub> were similar indicating that the mutation did not affect the xylanases' preference for WU-AX over WE-AX. However, for BsX, the addition of TAXI lead to a higher degree of inhibition when incubated with WE-AX than with WU-AX. Some evidences suggest that the presence of surface exposed aromatic residues can play a role in xylanase substrate selectivity (Moers et al., 2007). This subject is certainly worth exploring further.



# Kinetics and substrate selectivity of a *Triticum aestivum* xylanase inhibitor (TAXI) resistant D11F/R122D variant of *Bacillus subtilis* XynA xylanase

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## ABSTRACT

This study examined the kinetics and substrate selectivity of a GH11 *Bacillus subtilis* XynA xylanase (BsX) sensitive to inhibition by TAXI and an engineered variant, which is much less inhibited by TAXI (BsX<sub>mut</sub>). The main purpose of the work was to elucidate any influence of the structural point mutations on the kinetics and substrate selectivity of the enzyme. Three-dimensional structures of both xylanases were superimposed to elucidate the structural basis for differences in their hydrolytic properties. The two xylanases were incubated individually with water-extractable arabinoxylan (WEAX), water-unextractable arabinoxylan (WUAX), birchwood xylan, and wheat bran. Both the BsX and the BsX<sub>mut</sub> catalyzed the release of xylo-oligosaccharides with higher degree of polymerization from WUAX than from WEAX. At equimolar addition levels the activity of the BsX<sub>mut</sub> was lower than that of the BsX with respect to both the initial rate and the product yields obtained after prolonged reaction on the xylan substrates. The calculated substrate selectivity factors indicated that the BsX and the BsX<sub>mut</sub> both had higher catalytic rate on WUAX than on WEAX. Addition of a 100:1 (TAXI:xylanase) molar ratio of inhibitor confirmed the significantly decreased inhibition of BsX<sub>mut</sub> by TAXI. Addition of TAXI also influenced the xylanases' selectivity factor differently.

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## 1. Introduction

Enzymatic hydrolysis of xylans is of considerable importance in several cereal processes ranging from food and biotechnological applications to exploitation of xylans as a carbohydrate source for fermentation of xylose to biofuels or as a base for the development of novel prebiotic food ingredients (Courtin and Delcour, 2002; Bedford, 2003; Van der Borgh et al., 2005; Sørensen et al., 2006, 2007; Pastell et al., 2009). Xylan is a highly branched heteropolysaccharide which varies in structure between different plant species. It consists of a backbone of  $\beta$ -1,4 linked D-xylopyranosyl residues and depending on the source of the xylan and the procedure used in its extraction, the xylose units can be substituted at either the C(O)-2 or C(O)-3 or be di-substituted at both the C(O)-2- and the C(O)-3 position with L-arabinofuranose. The L-arabinofuranosyl side chain residues can also be esterified with ferulic or *p*-coumaric acid. Besides arabinose, the xylose units can be substituted with 4-O-methyl glucuronic acid residues (only at the C(O)-2 position) or they can be esterified with acetic acid (Ferreira-Filho, 1994).

Endo-1,4- $\beta$ -xylanases (xylanases, EC 3.2.1.8) catalyze the hydrolysis of the  $\beta$ -1,4 linkages in the arabinoxylan backbone, generating a mixture of xylo-oligosaccharides (Biely, 1985). Xylanases are mainly categorized in glycoside hydrolase (GH) families 10 and 11, although some xylanases are classified in GH5, GH8 and GH43 (Cantarel et al., 2009). All reported structures of GH11 xylanases have been described as a partially closed right hand consisting of only one domain folding into two  $\beta$ -sheets, which form a cleft on which the active site is situated (Collins et al., 2005). The cleft-shaped active site of GH 11 xylanases results in a restricted catalytic behavior where substituents or  $\beta$ -1,3 linkages represent a hindrance to the enzymes' activity and the GH11 xylanases are therefore most active on long chain xylan structures (Biely et al., 1997).

About 10 years ago, it was discovered that cereals such as wheat, durum wheat, barley and rye contained proteinaceous xylanase inhibitors which may affect the functionality of microbial xylanases, notably GH11 xylanases (Debyser et al., 1997; Rouau and Surget, 1998). By now, three distinct types of such xylanase inhibitors have been identified in cereals: TAXI (*Triticum aestivum* xylanase inhibitor), XIP (xylanase-inhibiting protein) and TL-XI (thaumatin-like xylanase inhibitor) (Croes et al., 2008). TAXI-type inhibitors are proteins of ~40 kDa that specifically inhibit bacterial and fungal GH11 xylanases (Gebruers et al., 2001). Based on struc-

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tures of TAXI in complex with different GH11 xylanases it has been shown that TAXI interacts reversibly with the active site region of the enzyme in which several amino acid residues of TAXI bind to the active site cleft of the enzyme. This binding prevents the access and binding of the xylan substrate in the active site of the enzyme (Sansen et al., 2004). Additionally, interactions of all three types of xylanase inhibitors with (arabino)xylans have been described (Fierens et al., 2008). The affinity of the xylanase inhibitors for the (arabino)xylans generally increase with decreasing A/X ratios. This means that the interactions between the inhibitor and the xylan chains manifest themselves more when only a low degree of – or no – substitutions are present on the xylan.

Because of the increased significance of microbial xylanases in baking and other cereal processing applications (Debyser et al., 1997; Courtin and Delcour, 2002), the problem of xylanase inhibitors reducing the activity of added microbial xylanases is significant (Sørensen et al., 2004; Trogh et al., 2004; Frederix et al., 2004; Courtin et al., 2005). In order to counteract this problem, a *Bacillus subtilis* XynA xylanase (BsX) (GH11) was recently modified to diminish the xylanase inhibitor interaction – particularly the xylanase-TAXI interaction (Sørensen and Sibbesen, 2006). BsX was chosen as a template since this bacterial xylanase is inhibited by TAXI but unaffected by XIP, which is specific for fungal xylanases, and TL-XI (Debyser et al., 1997; McLauchlan et al., 1999; Fierens et al., 2007). Fifty-seven catalytically active variants of BsX, each containing one point mutation, were evaluated based on specific activity and inhibitor sensitivity against TAXI and XIP. Based on the obtained results, the D11F/R122D variant, which is studied in the present work, was selected for further use. This enzyme is now used commercially, sold as GRINDAMYL™ POWERBake (Danisco A/S, Brabrand, Denmark). In this variant the amino acid residues participating in catalysis or situated inside the active site cleft were not mutated in order to avoid affecting the catalytic activity of the enzyme. Instead, surface residues, D11 (residue 11, aspartic acid) and R122 (residue 122, arginine), surrounding the active site cleft, were mutated to F (phenylalanine) and D (aspartic acid), respectively. The single R122D mutation was found to increase the activity of the xylanase, but did not induce a significant resistance to inhibition by TAXI, whereas the D11F mutation produced a significant resistance to inhibition by TAXI (Sørensen and Sibbesen, 2006). The exact influence of the double mutation D11F and R122D on the activity of the mutated enzyme on various xylan and arabinoxylan substrates and on the enzyme's selectivity towards water-unextractable arabinoxylan (WUAX) versus water-extractable arabinoxylan (WEAX), respectively, has not been investigated, however.

The purpose of the present study was therefore to evaluate the specificity and the TAXI resistance of this engineered TAXI resistant variant of the *B. subtilis* XynA xylanase (BsX<sub>mut</sub>) and compare its performance to that of the wildtype *B. subtilis* XynA xylanase on different substrates. This was done by adding TAXI to various xylan substrates in order to mimic the conditions in genuine reaction media such as e.g. wheat dough where the xylanase will encounter the inhibitor. In this study we report on product profiles, catalytic rates and substrate selectivity factors of the enzymes. In order to elucidate the structural basis responsible for the different substrate specificities of the wildtype and recombinant xylanase, a comparison of three-dimensional structures was also performed.

## 2. Materials and methods

### 2.1. Chemicals

All reagents were of at least analytical grade and supplied by Sigma–Aldrich (Bornem, Belgium), unless specified otherwise.

Xylo-oligosaccharides (X2–6) from birchwood xylan were purchased from Megazyme (Bray, Ireland).

### 2.2. Substrates

Water-unextractable arabinoxylan (WUAX) (arabinose:xylose (A/X): 0.61) and water-extractable arabinoxylan (WEAX) medium viscosity (A/X: 0.61), both from wheat were obtained from Megazyme (Bray, Ireland). Birchwood xylan was purchased from Sigma–Aldrich (Bornem, Belgium) and used as it is. According to the manufacturer the xylose content of this substrate was more than 90%. WEAX and birchwood xylan were solubilized with ethanol as recommended in the product sheet. Wheat bran was a commercial product from Natur Drogeriet A/S (Hørning, Denmark). The wheat bran was destarched using a modified version of Merck “Total Dietary Fiber” measurement kit (12979 Bioquant) without protease treatment. The wheat bran was treated sequentially with  $\alpha$ -amylase (Termamyl SC) (95 °C, pH 6, 60 min) and amyloglucosidase (Spirizyme Plus FG) (60 °C, pH 4.5, 30 min) followed by ethanol precipitation. Both enzymes used were obtained from Novozymes A/S (Bagsværd, Denmark). Acid hydrolysis (0.4 M HCl, 2 h, 100 °C) (Sørensen et al., 2003) of wheat bran showed that the monosaccharide composition was 3 wt% galactose, 10 wt% glucose, 32 wt% arabinose and 55 wt% xylose (measured by HPAEC according to Arnous and Meyer, 2008).

### 2.3. Enzymes

The wildtype BsX enzyme, sensitive to inhibition by TAXI but not to XIP, and the variant hereof, BsX<sub>mut</sub>, less prone to inhibition by either TAXI or XIP, were obtained from Danisco A/S (Brabrand, Denmark). BsX was purified from a GRINDAMYL™ H 640 enzyme preparation and carries a Swiss Prot entry P18429, XynA.BACSU. BsX<sub>mut</sub> was purified from a GRINDAMYL™ POWERBake enzyme preparation. The enzyme was obtained by site-directed mutagenesis and differs from BsX only in two amino acids: D11F and R122D (Sørensen and Sibbesen, 2006). Both enzymes were free from side activities and were purified according to Sørensen and Sibbesen (2006). Both xylanases had a molecular weight of 23 kDa, pH optimum at 6, temperature optimum at 50 °C, and had similar pH and temperature stabilities. They have been classified in glycosyl hydrolase family 11 according to sequence homology (Cantarel et al., 2009).

### 2.4. Inhibitor

TAXI was obtained from Danisco A/S (Brabrand, Denmark) as a purified mixture of TAXI I and TAXI II (Gebruers et al., 2001). The inhibitor was solubilized in 0.1 M sodium acetate buffer pH 6.

### 2.5. Enzyme structure comparison

Since no structure of BsX has been published, the structure of *Bacillus circulans* endo- $\beta$ -1,4-xylanase (GH11) (Swiss Prot entry P09850) served as a model for the BsX structure. The *B. circulans* xylanase and the BsX are identical, except for residue 147 situated on the opposite side of the active site. The S147 in BsX corresponds to T147 in the *B. circulans*. Coordinates for the structure of *B. circulans* xylanase A (PDB ID: 1XNB, resolution: 1.49 Å, R-factor: 0.165) (Campbell and Bedford, 1992) and the BsX<sub>mut</sub> (PDB ID: 2B45, resolution: 2.00 Å, R-factor: 0.162) (Sansen et al., 2004) were downloaded from the RCSB Protein Data Bank ([www.rcsb.org/pdb](http://www.rcsb.org/pdb)). The molecular structures were visualized using the program PyMOL (v0.99) (DeLano Scientific, San Carlos, CA).

## 2.6. Enzymatic hydrolysis of xylan substrates

Enzyme catalyzed hydrolysis, using BsX and BsX<sub>mut</sub> respectively, of the four substrates (WEAX, WUAX, birch wood xylan and wheat bran) was assessed according to the following procedure: substrate (1 wt%) was dissolved in 0.1 M sodium acetate pH 6 with 0.02 wt% sodium azide. The substrate was preincubated for 10 min at 40 °C under continuous stirring before a suitably diluted enzyme solution was added (to give a final reaction concentration of 0.12 μmole enzyme g<sup>-1</sup> dry matter (DM) substrate). Samples were withdrawn after 0 h, 0.5 h, 1 h, 2 h, 4 h, 18 h and 24 h and the enzymatic hydrolysis was stopped immediately by heating the samples at 100 °C for 10 min. After centrifugation (10,000 × g, 10 min), the supernatant was analyzed for xylo-oligosaccharides by HPAEC (see later). The procedures were performed in triplicate for both enzymes and a reference (no enzyme). For inhibition studies 110 nmole TAXI per nmole xylanase was preincubated with the substrates (WEAX and WUAX) prior to addition of the xylanases.

To evaluate the initial catalytic reaction rates ( $v_i$ ) of BsX and BsX<sub>mut</sub> on the four substrates, initial slopes of progress curves were calculated from extended hydrolysis reactions. Furthermore, the initial rate was calculated for each of the enzyme catalyzed reactions on WEAX and WUAX, in the presence of TAXI.

The relative activity of a xylanase towards WUAX and WEAX is referred to as the substrate selectivity factor (SSF) (Moers et al., 2003). The substrate selectivity factors for BsX and BsX<sub>mut</sub> respectively, were calculated based on  $v_i$  and the maximal yields obtained after 24 h of incubation ( $Y_{MAX}$ ). The SSF( $v_i$ ) therefore describes the enzyme's relative preference for WUAX versus WEAX based on the difference in initial rates, whereas the SSF( $Y_{MAX}$ ) expresses the relative difference between the extent of degradation of the two substrates with respect to the final yields. In Moers et al.'s (2003) the SSF was based on enzyme activity after 1 h incubation. However, in this study the SSF was based on incubation periods of 0.5 h (SSF( $v_i$ )) and 24 h (SSF( $Y_{MAX}$ )), respectively, since a more in depth characterization of the xylanase activities was wanted regarding the initial and endpoint substrate selectivity.

$$SSF(Y_{MAX}) = \frac{Y_{MAX}(WUAX)}{Y_{MAX}(WEAX)}, \quad SSF(v_i) = \frac{v_i(WUAX)}{v_i(WEAX)}$$

In the presence of TAXI, the SSF( $Y_{MAX, TAXI}$ ) and SSF( $v_{i, TAXI}$ ) were calculated in the same way. A high SSF thus corresponds to a relatively high preference of the xylanase for catalyzing solubilization of WUAX, while a low such factor indicates a relative preference of the enzyme for catalyzing the hydrolysis of WEAX.

## 2.7. High performance anion exchange chromatography (HPAEC)

Separation and quantification of xylo-oligosaccharides in the hydrolysates were performed using an ICS3000 system consisting of GS50 gradients pumps/ED50 electrochemical detector/AS50 chromatography compartment coupled to an AS50 autosampler (Dionex Corp., Sunnyvale, CA). Samples (25 μl) were injected on a CarboPac™ PA1 (4 mm × 250 mm) analytical column (Dionex Corp., Sunnyvale, CA) at a flow rate of 1 ml min<sup>-1</sup>. Complete separation of xylo-oligosaccharides was achieved principally according to the method of Katapodis et al. (2002) but with modifications using a three-eluent system comprising deionised water (18.2 mΩ cm at 25 °C) (A), 0.5 M NaOH (B) and 0.5 M NaOAc (C). Xylo-oligosaccharides were separated using the following linear gradient program: 0 min, 80:20:0 (% A:B:C); 5 min, 70:20:10; 20 min, 70:20:10. Strongly retained anions were removed from the column by increasing (B) to 80% for 10 min followed by reequilibration of the column where (B) was reduced to 0% for 10 min. During chromatography, the eluents were kept under a blanket of nitrogen and the mobile phase was purged with nitrogen to min-

imize carbonate contamination, which would affect the retention time of the xylo-oligosaccharides. The quantification was carried out by use of external standards. The following pulse potentials and durations were used for detection: E1 = 0.1 V, t1 = 400 ms; E2 = -2 V, t2 = 20 ms; E3 = 0.6 V, t3 = 10 ms; E4 = -0.1 V, t4 = 70 ms, and data collection rate = 0.2 Hz. Data were collected and analyzed on computers equipped with Chromeleon 6.80 Sp2 Build 1472 software (Dionex Corp., Sunnyvale, CA).

## 3. Results

### 3.1. Enzyme structure comparison

To investigate the structural basis for differences in the catalytic properties of BsX and BsX<sub>mut</sub>, the three-dimensional structure of BsX was modeled using the *B. circulans* xylanase A (PDB ID: 1XNB) (Campbell and Bedford, 1992) as the search model. The model of BsX was superimposed onto the BsX<sub>mut</sub> model (PDB ID: 2B45) (Sansen et al., 2004) in order to elucidate the structural change of the D11F and R122D mutation. The two available *B. subtilis* xylanase structures with PDB entry 2Z79 (*B. subtilis* xylanase soaked with xylotetraose) and 2QZ3 (*B. subtilis* xylanase soaked with xylotetraose), respectively, are both E172A mutants and hence not structures of the wildtype BsX, and could therefore not be used to assess the BsX point mutations.

As seen in Fig. 1 the D11F mutation appears to cause a slight narrowing of the entrance to the active site cleft because the phenylalanine is more bulky than the aspartic acid. Apparently the R122D mutation may have the opposite effect since the change of arginine to aspartic acid causes a slight widening of the entry to the active site cleft.

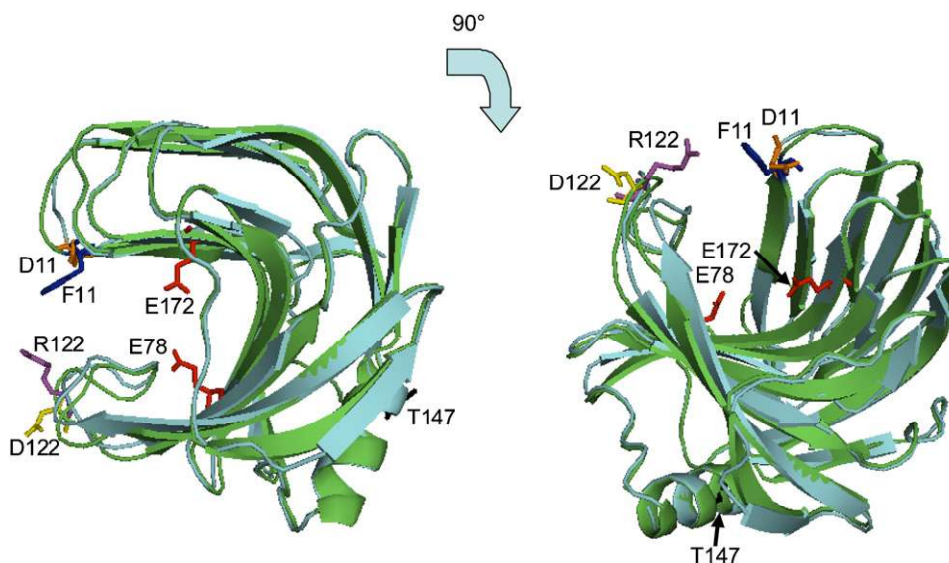
### 3.2. Substrate specificity

#### 3.2.1. Activity of BsX and BsX<sub>mut</sub> on WEAX and WUAX with and without TAXI

Analysis of the activity of BsX and BsX<sub>mut</sub> on WEAX and WUAX revealed that both enzymes catalyzed the generation of significant amounts of small xylo-oligosaccharides (DP 2–6). From both substrates the main products were xylobiose (X2), xylotriose (X3) and to some extent xylotetraose (X4) during a 24 h incubation (Fig. 2). For BsX the main difference between the results of the enzymatic catalysis on the two substrates was that in total about 25% more xylo-oligosaccharides were solubilized from WUAX than from WEAX (Fig. 2(A) and (B)). This difference was mainly contributed by higher levels of X3 and X4 from WUAX than from WEAX. Activity of BsX<sub>mut</sub> during the whole course of reaction was somewhat reduced by the double mutation D11F/R122D since the enzyme had lower activity on both WEAX and WUAX as compared to the BsX (Fig. 2). Again, higher levels of X3 and X4 were released from WUAX as compared to from WEAX.

In the presence of TAXI the levels of both X3 and XOS catalytically released by BsX from WEAX, were decreased by 37% and X2 by 47%, respectively, as compared to the corresponding reaction without TAXI (Figs. 2(A) and 3(A)). The XOS levels generated from WUAX in the presence of TAXI were decreased by 26%, X2 by 29% and X3 by 28% (Fig. 3(B)). The relative decreases in the BsX activity on the WEAX and WUAX in the presence of TAXI were thus relatively similar (or at least of the same order of magnitude), although the higher relative decrease in the X2 liberation on WEAX indicated that the BsX activity might be relatively more inhibited by TAXI when acting on WEAX than on WUAX. The same tendency was observed in the initial velocities which were obtained directly from the initial slopes of the progress curves obtained during the first 30 min (Table 1). The initial velocity of the hydrolysis of WEAX was more affected by addition of TAXI than the initial velocity data





**Fig. 1.** Superposition of the *B. circulans* xylanase (green) and D11F/R122D variant ( $BsX_{mut}$ ) (light blue) visualizing the mutation of the amino acid, D11 (orange) and R122 (purple) in the *B. subtilis* XynA xylanase wildtype ( $BsX$ ), which has been mutated to F (dark blue) and D122 (yellow) respectively in the variant  $BsX_{mut}$ . The two catalytic glutamic acid residues (E78 and E172) are highlighted in red. The residue highlighted in black (T147) is threonine in *B. circulans* xylanase but serine (S147) in  $BsX$ . This figure was drawn using PyMOL (v0.99) (DeLano Scientific, San Carlos, CA) based on the PDB-file 1XNB of *B. circulans* (Campbell and Bedford, 1992) and 2B45 of *B. subtilis* (Sansen et al., 2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

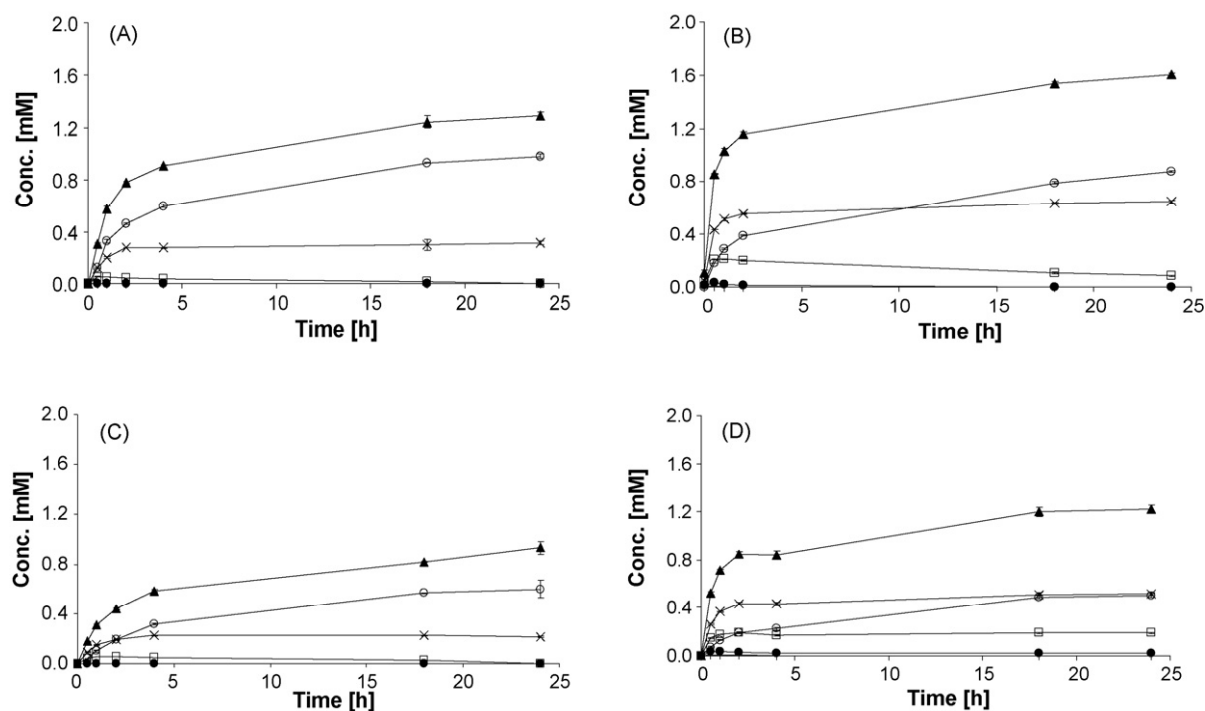
on WUAX since the initial rates with TAXI addition decreased by approximately 46% on WEAX and 30% on WUAX.

$BsX_{mut}$  was selected because of its insensitivity to inhibition by TAXI. The  $BsX_{mut}$  was only inhibited to a relatively low extent: from WEAX the relative decrease in XOS liberation was equivalent to 5%, and X2 and X3 10% and 7%, respectively, of the levels obtained without TAXI addition (Figs. 2(C) and 3(C)). When WUAX was incubated with  $BsX_{mut}$  the enzymatically liberated XOS levels were decreased by only 3% and X3 by 0.2%, whereas the levels of X2 were increased

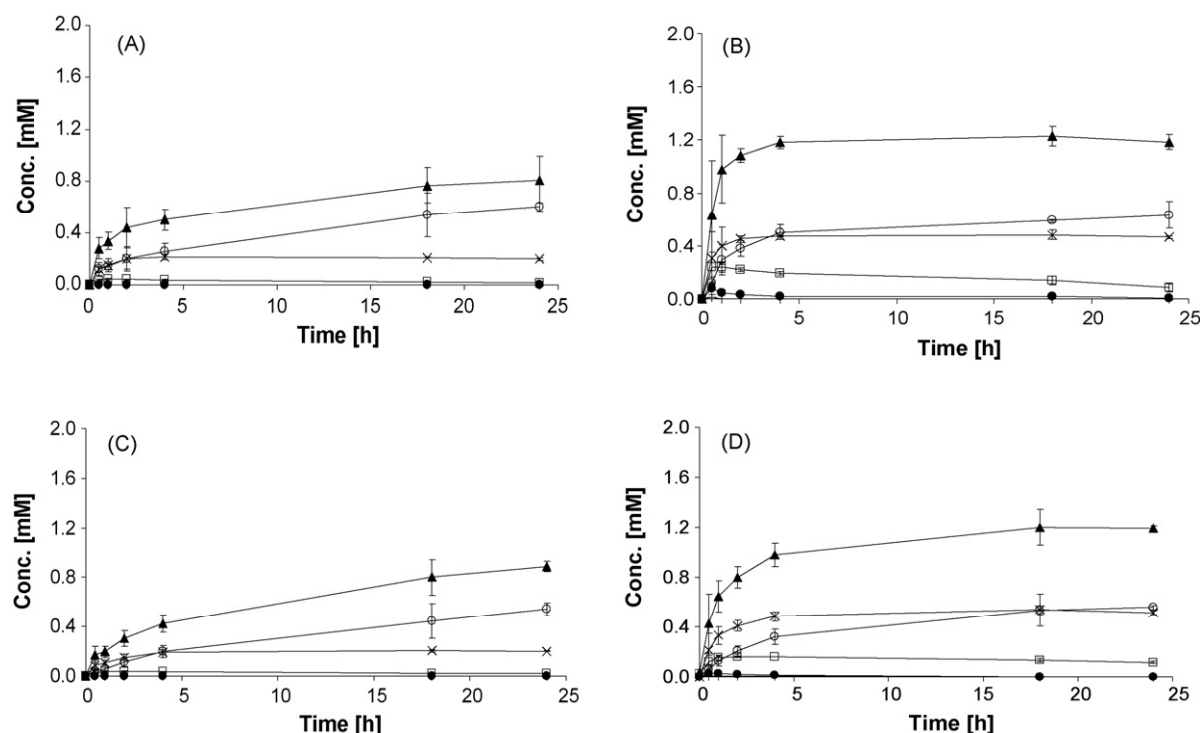
by 11% (Figs. 2(D) and 3(D)). Furthermore, the initial rates showed no significant effect of TAXI on WEAX and on WUAX the catalytic rate was decreased by approximately 16% (Table 1). The data thus confirmed the very low extent of inhibition of  $BsX_{mut}$  when TAXI was added to WEAX and WUAX.

### 3.2.2. Activity of $BsX$ and $BsX_{mut}$ on different xylan substrates

In order to evaluate the mode of action of  $BsX$  and  $BsX_{mut}$  on other xylanolytic substrates the outcomes of enzymatic treat-



**Fig. 2.** HPAEC product analysis of  $BsX$ : (A) WEAX and (B) WUAX,  $BsX_{mut}$ : (C) WEAX and (D) WUAX. The enzymes ( $0.12 \mu\text{mole g}^{-1}$  DM substrate) were incubated with 1% WEAX or WUAX in sodium acetate buffer (0.1 M) of pH 6 at 40 °C. ○: xylobiose, ×: xylotriose, □: xylo-tetraose, ●: xylo-pentaose, ▲: xylo-oligosaccharides (DP 2–6). Values represent the mean of three independent experiments and shown  $\pm 1$  standard deviation.



**Fig. 3.** HPAEC product analysis of BsX and BsX<sub>mut</sub> in the presence of TAXI. BsX: (A) WEAX + TAXI and (B) WUAX + TAXI, and BsX<sub>mut</sub>: (C) WEAX + TAXI and (D) WUAX + TAXI. Each enzyme (0.12  $\mu\text{mole g}^{-1}$  DM substrate) was incubated with 1% WEAX or WUAX respectively in sodium acetate buffer (0.1 M) of pH 6 at 40 °C. 110 nmole TAXI per nmole xylanase was added to the substrate for inhibition experiments. (○): Xylobiose; (×): Xylotriose; (□): Xylotetraose; (●): Xylopentose; (▲): Xylo-oligosaccharides (DP 2–6). Values represent the mean of three independent experiments and shown  $\pm 1$  standard deviation.

ment were measured on birchwood xylan, that had a relatively lower substitution degree than WEAX and WUAX, and on wheat bran, which was a more complex substrate. Both xylanases catalyzed a higher release of XOS from birchwood xylan and had higher initial rates than when acting on WEAX and WUAX (Table 1, Fig. 4(A) and (C)). This result may be related to the relatively un-substituted structure of the birchwood xylan. In accordance with the hydrolysis results obtained with WEAX and WUAX, BsX had higher activity than BsX<sub>mut</sub> and both xylanases also catalyzed the release of primarily X2–4 from birchwood xylan.

When wheat bran was subjected to treatment with BsX and BsX<sub>mut</sub>, respectively, lower levels of XOS were produced as compared to the levels obtained on WEAX, WUAX and birchwood xylan with both xylanases (Fig. 4(B) and (D)). Analogously, the lowest initial rates were obtained on the wheat bran (Table 1). However, the initial rates for the hydrolysis of wheat bran were quite similar for BsX and BsX<sub>mut</sub> and were similar to those obtained by BsX<sub>mut</sub> on WEAX.

### 3.3. Substrate selectivity

In contrast to substrate specificity which describes the ability of an enzyme to distinguish one substrate from similar substrates, the relative activity of a xylanase towards WUAX and WEAX is referred to as substrate selectivity and calculated as the substrate selectivity factor (SSF) (Moers et al., 2003). Two different SSF have been calculated: SSF( $Y_{\text{MAX}}$ ) was calculated based on the maximal yields obtained after 24 h of hydrolysis presented in Figs. 2 and 3. SSF( $v_i$ ) was calculated based on the initial rates presented in Table 1. Substrate selectivity factors were calculated for the hydrolysis reactions of both enzymes in the absence and in the presence of TAXI.

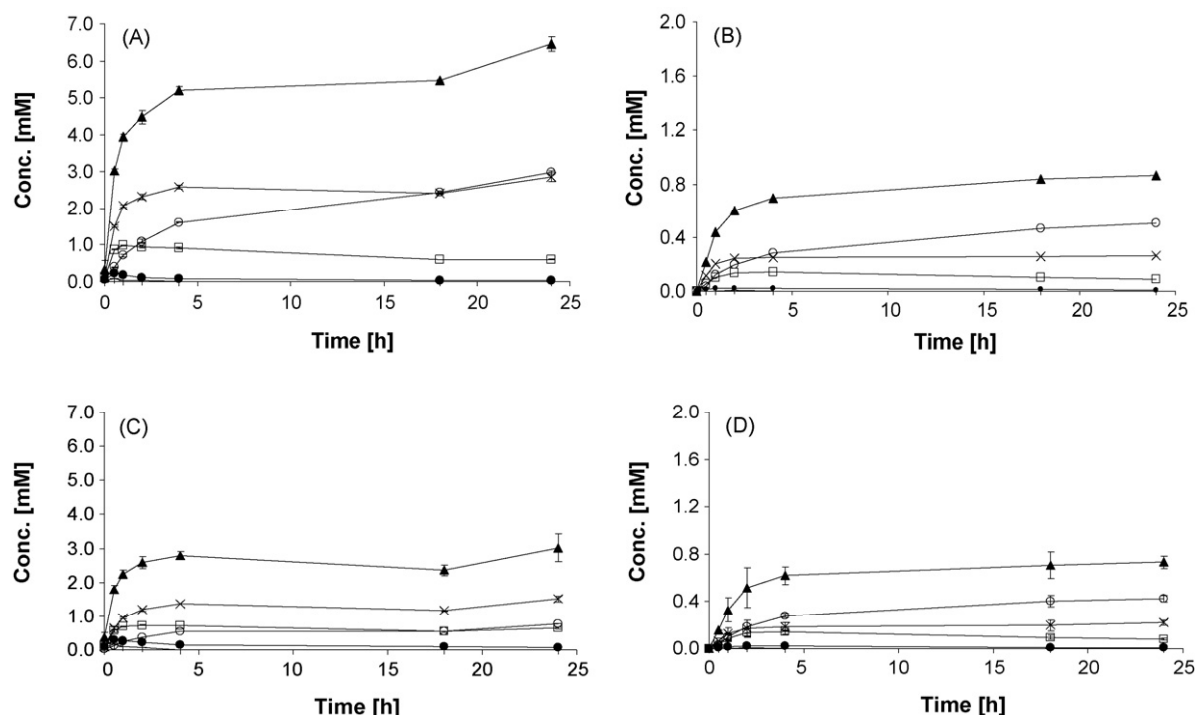
The data in Table 2 show that all the calculated SSF values were in the same range (0.89–3.08) for both enzymes. Most of the SSF values were larger than one, which indicates that both xylanase preferentially hydrolyses WUAX over WEAX. This is also seen from Figs. 2 and 3 where higher concentrations of xylo-oligosaccharides were produced from WUAX than from WEAX. However for the

**Table 1**

Initial rates ( $v_i$ ) [ $\mu\text{M min}^{-1}$ ] from the hydrolysis of individual polymeric substrates by BsX and BsX<sub>mut</sub> of various XOS. Coefficients of variation were all < 10.4%.

	WEAX	WEAX + TAXI	Inhibition [%]	WUAX	WUAX + TAXI	Inhibition [%]	Birchwood xylan	Wheat bran
<b>BsX</b>								
X2	7.23 <sup>d,x</sup>	4.01 <sup>b,y</sup>	44.5	5.93 <sup>b,x</sup>	3.99 <sup>b,y</sup>	32.8	12.75	1.21
X3	7.85 <sup>e,x</sup>	3.97 <sup>d,y</sup>	49.4	14.47 <sup>d,x</sup>	10.23 <sup>d,y</sup>	29.3	49.86	3.85
XOS	16.92 <sup>f,x</sup>	9.26 <sup>f,y</sup>	45.3	28.60 <sup>f,x</sup>	21.00 <sup>f,y</sup>	26.6	100.9	7.26
<b>BsX<sub>mut</sub></b>								
X2	1.38 <sup>a,x</sup>	1.36 <sup>a,x</sup>	1.4	2.39 <sup>a,x</sup>	2.07 <sup>a,x</sup>	13.4	3.88	1.00
X3	3.01 <sup>b,x</sup>	2.84 <sup>c,x</sup>	5.6	8.65 <sup>c,x</sup>	7.12 <sup>c,x</sup>	17.7	21.31	2.17
XOS	5.65 <sup>c,x</sup>	5.73 <sup>e,x</sup>	−1.5	17.40 <sup>e,x</sup>	14.29 <sup>e,y</sup>	17.9	60.05	5.49

Results in the same column followed by different superscript letters a–f are significantly different at  $P < 0.05$ . Results in the same row, pair wise for SSF( $Y_{\text{MAX}}$ ) and SSF  $v_i$ , followed by different superscript letters x, y are significantly different at  $P < 0.05$ .



**Fig. 4.** Progress curves of the products generated from birchwood xylan and wheat bran hydrolysis by BsX and BsX<sub>mut</sub> respectively. (A) Birchwood xylan + BsX, (B) wheat bran + BsX, (C) birchwood xylan + BsX<sub>mut</sub>, and (D) wheat bran + BsX<sub>mut</sub>. Each enzyme (0.12  $\mu\text{mole g}^{-1}$  DM substrate) was incubated with 1% birchwood xylan or wheat bran in sodium acetate buffer of pH 6 at 40 °C. (○): Xylobiose; (×): Xylotriiose; (□): Xylo-tetraose; (●): Xylopentaose; (▲): Xylo-oligosaccharides (DP 2–6). Values represent the mean of three independent experiments and are shown  $\pm 1$  standard deviation.

**Table 2**  
Selectivity factors for BsX and BsX<sub>mut</sub>.

	SSF( $Y_{\text{MAX}}$ )	SSF( $Y_{\text{MAX, TAXI}}$ )	$\Delta\text{SSF}(Y_{\text{MAX}})$ [%]	SSF( $v_i$ )	SSF( $v_i, \text{TAXI}$ )	$\Delta\text{SSF}(v_i)$ [%]
BsX						
X2	0.89 <sup>a,x</sup>	1.22 <sup>b,y</sup>	36.8	0.82 <sup>a,x</sup>	0.99 <sup>a,x</sup>	21.2
X3	2.08 <sup>c,x</sup>	2.37 <sup>d,y</sup>	13.7	1.84 <sup>e,x</sup>	2.58 <sup>c,x</sup>	39.8
XOS	1.24 <sup>b,x</sup>	1.46 <sup>c,y</sup>	17.6	1.69 <sup>c,x</sup>	2.27 <sup>b,x</sup>	34.2
BsX <sub>mut</sub>						
X2	0.83 <sup>a,x</sup>	1.03 <sup>a,y</sup>	24.1	1.73 <sup>b,x</sup>	1.52 <sup>a,x</sup>	−12.1
X3	2.43 <sup>d,x</sup>	2.59 <sup>e,y</sup>	6.8	2.87 <sup>f,x</sup>	2.51 <sup>c,x</sup>	−12.8
XOS	1.31 <sup>b,x</sup>	1.34 <sup>c,x</sup>	1.8	3.08 <sup>d,x</sup>	2.49 <sup>b,x</sup>	−19.0

Results in the same column followed by different superscript letters a–f are significantly different at  $P < 0.05$ . Results in the same row, pair wise for SSF( $Y_{\text{MAX}}$ ) and SSF( $v_i$ ), followed by different superscript letters x, y are significantly different at  $P < 0.05$ .

release of X2, the SSF was lower than 1 (except for the SSF( $v_i$ ) of the BsX<sub>mut</sub>) indicating that lower levels of X2 were produced from WUAX than from WEAX. When the data for the two xylanases were compared there were no significant differences in SSF( $Y_{\text{MAX}}$ ) (except for X3). However the SSF( $v_i$ ) values were relatively higher for the BsX<sub>mut</sub> than for the BsX. This difference indicates that the variant had a relatively higher catalytic rate on WUAX than on WEAX. As expected, the addition of TAXI had the highest impact on the SSF( $v_i$ ) for BsX since the average increase in the SSF( $v_i$ ) was 32% which was higher than the average increase of the SSF( $Y_{\text{MAX}}$ ) (23%). Again, this result indicates that BsX is more sensitive to inhibition by TAXI when incubated with WEAX than WUAX. For BsX<sub>mut</sub>, the activities on WEAX and WUAX were not significantly changed by addition of TAXI as the SSF( $Y_{\text{MAX, TAXI}}$ ) was only 11% higher on average than the SSF( $Y_{\text{MAX}}$ ) (Table 2). SSF( $v_i$ ) for BsX<sub>mut</sub> actually decreased upon addition of TAXI which means that the initial activity of BsX<sub>mut</sub> on WUAX was decreased more than on WEAX when TAXI was added.

#### 4. Discussion

BsX and the BsX<sub>mut</sub> displayed typical endo-activity against all four substrates since a mixture of xylo-oligosaccharides was generated during the hydrolysis and no significant amounts of xylose were measured in any of the hydrolysates. This is a common characteristic of GH11 xylanases from *A. niger* (Meagher et al., 1988), *Schizophyllum commune* (Bray and Clarke, 1992), *Penicillium funiculosum* and *P. griseofulvum* (Berrin et al., 2007) that all yield X2 and X3 as main end reaction products. The present study does not allow us to explain why X2 and X3 were released in the highest concentrations. However, previous results have shown that incubation of WEAX and WUAX with increasing dosages of a BsX gradually results in a shift in apparent molecular weight of the formed arabinoxylan fragments to a lower molecular weight (Moers et al., 2005). The relatively high enzyme dosage (0.12  $\mu\text{mole g}^{-1}$  DM substrate) and long reaction times used in the present study might thus explain the high releases of X2 and X3.



The catalytic effects and rates of both xylanases on substrates that differ in complexity may be related to the general structure of the active site for GH11 xylanases. In this family, the cleft-shaped active site results in a restricted catalytic behavior where substituents present a hindrance (Biely et al., 1997). Since birchwood xylan has a lower degree of substitution than the other substrates used, the birchwood xylan – or rather stretches hereof – may be better accommodated into the active site cleft than e.g. wheat bran arabinoxylan which has a relatively more complex structure. Furthermore, Bonnin et al. (2006) showed that the arabinose content of the substrate affected the activity of BsX in a linear manner related to the arabinose:xylose ratio: With increasing arabinose substitutions the activity of BsX decreased.

The reduced activity of the BsX<sub>mut</sub> compared to BsX on all four substrates (WEAX, WUAX, birchwood xylan and wheat bran) was in accordance with previous findings (Sørensen and Sibbesen, 2006). The present experimental set-up and the analyses conducted do not allow any firm conclusions to be drawn as to whether the small differences in the product yields of the two enzymes were due solely to the introduction of a more bulky amino acid at the entrance to the active site of the enzyme – presumably narrowing the entrance of substrate to the active site – or a result of another rate retarding feature, e.g. altered molecular flexibility, of the BsX<sub>mut</sub>.

Previous results have shown that a R122D mutation in BsX increased the activity by 31% (Sørensen and Sibbesen, 2006). Furthermore, crystallographic analysis of the structure of *B. subtilis* xylanase A mutants has recently revealed that the thumb which contains residue R122 has a functional role in the catalysis since it is the most flexible part of this xylanase molecule (Pollet et al., 2009). The thumb's flexibility apparently plays a role in the open–close movements and therefore in the accessibility of the substrate to the enzyme. Therefore, the R122 mutation was combined with the D11F mutation in order to increase the activity of the less TAXI-inhibited xylanase (BsX<sub>mut</sub>), which it did (Sørensen and Sibbesen, 2006). However, in the present study, using different xylan substrates, the effect of this mutation on the activity of the enzyme was not apparent since the BsX<sub>mut</sub> still had slightly lower activity than BsX (Table 1).

In this study approximately 110 nmole TAXI per nmole xylanase was used to assess the effect of TAXI on the catalyzed hydrolysis by the two xylanases. Typical inhibitor concentrations in wheat flour are in the range 110 mg kg<sup>-1</sup> for TAXI (Bonnin et al., 2005). Generally, the molar xylanase inhibitor concentrations in wheat flours are much higher than the xylanase dosages used in biotechnological processes (Gebruers et al., 2005). Since the xylanase and the inhibitor form a 1:1 complex (Fierens et al., 2005; Sansen et al., 2004), it is intriguing that despite high inhibitor concentrations, xylanases are still catalytically active and can be used in biotechnological processes. A reason for this may be that the concentrations of both TAXI and xylanases are so low in genuine applications that there may be a delay in the complex formation as a result of the low probability that the TAXI and xylanase actually get in contact before the enzyme attacks the substrate; hence, the relative concentration of substrate to enzyme is much larger than the relative concentration of TAXI to enzyme, providing a favorable competitive advantage of the enzyme-to-substrate interaction as compared to the TAXI:xylanase complex formation. In addition, the inhibition by TAXI is reversible. Secondly, biotechnological applications of microbial xylanases do not fully resemble the biochemical *in vitro* experiments with respect to mobility, in many of the real applications (e.g. baking) water might for example limit the mobility of both enzyme and TAXI.

The SSF provides a valuable prediction of xylanase functionality in biotechnological applications since xylanases that differ in substrate selectivity, can be used in different applications. Both the SSF( $Y_{MAX}$ ) and SSF( $v_i$ ) were lower than previously reported for

BsX where a SSF for BsX of 22 (Moers et al., 2003) and 366 (Moers et al., 2005) have been reported. Furthermore Trogh et al. (2004) reported an SSF value of six for a variant (D11F/R122D/Q175L) of BsX. The difference in results reported here and by Moers et al. (2003, 2005) and Trogh et al. (2004) may be due to methodological differences as the results in the present study were a result of an initial rate and an endpoint measurement, whereas the results reported in previous studies have reflected the SSF's at a certain timepoint. Furthermore, differences in type and concentration of the substrates, methods for activity measurements and the incubation period may also explain the differences between our data and the data by Moers et al. (2003, 2005) and Trogh et al. (2004). In the present study SSF( $Y_{MAX}$ ) and SSF( $v_i$ ) were based on product concentrations (X2, X3 and XOS) released from 10 mg ml<sup>-1</sup> WEAX and WUAX substrates obtained from Megazyme. The SSF( $v_i$ ) was calculated based on initial rates after 0.5 h incubation and SSF( $Y_{MAX}$ ) on maximal yields after 24 h incubation. In Moers et al.'s (2003) and Trogh et al.'s (2004) the BsX and the variant were incubated with 5 mg ml<sup>-1</sup> chromophoric arabinoxylan substrates for 1 h and the SSF was calculated based on reducing end measurements. The SSF in Moers et al. (2005) was obtained when the BsX was incubated with 5 mg ml<sup>-1</sup> native substrates (WEAX and WUAX extracted from wheat flour) for 15 min. The SSF was based on the ratio of specific solubilizing activity upon incubation of WUAX based on solubilized xylose and the specific enzyme activity towards WEAX was based on the formation of reducing ends. These methodological differences may explain the large difference in SSF values between our data and previously reported data (Moers et al., 2003, 2005; Trogh et al., 2004).

Addition of TAXI affected the SSF since an increase was observed. For this inhibition study an increase in SSF indicated a larger inhibition in the hydrolysis of WEAX than of WUAX. This might be due to the water solubility of TAXI that may provide for a faster interaction with the soluble substrate – this is not a direct enzyme inhibition, but manifests as inhibition of the enzymatic reaction. It cannot be excluded that the sensitivity difference is a result of complex differences in the kinetics of the enzyme attack on WEAX as compared to on WUAX, but it is tempting to conclude, that the higher sensitivity of BsX on WEAX than on WUAX is due to their difference in water solubility. Since the binding affinity of TAXI increases with decreasing arabinose:xylose ratio (Fierens et al., 2008), another hypothesis could be that TAXI had a higher binding affinity for one of the substrates and therefore inhibited the xylanase to a lower degree on that substrate. However, despite the similar A/X ratio among WEAX and WUAX it cannot be excluded that the arabinose substitution pattern differed, and that this difference among the substrates could influence the binding of TAXI, and in turn the level of unbound TAXI “available” for acting as an enzyme inhibitor.

## 5. Conclusions

This study established that the overall activity of the *B. subtilis* XynA variant (BsX<sub>mut</sub>) was reduced on different polymeric xylan substrates. Based on the structure analysis, the reduced activity of BsX<sub>mut</sub> might be due to the steric hindrance caused by the D11F mutation. The overall selectivity factors of BsX and BsX<sub>mut</sub> were similar indicating that the mutation did not affect the xylanases' preference for WUAX over WEAX. However, for BsX, the addition of TAXI led to a higher degree of inhibition when incubated with WEAX. Despite the slightly lowered catalytic rates and the small change in selectivity, the trade-off regarding insensitivity to TAXI inhibition was significant. The understanding of the changes in catalytic capabilities of mutant enzymes is very important for predicting enzyme dosages and enzyme catalysis outcome in genuine applications. The finding that one amino acid change at the entry to the active site did in fact alter the specificity may allow for new

protein enzyme design that can promote the enzyme catalyzed liberation of certain desirable product profiles in future applications.

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## **Chapter 4 Size exclusion chromatography for the quantitative profiling of the enzyme-catalyzed hydrolysis of xylo-oligosaccharides**

This chapter is extended in the form of Paper 3:

Size exclusion chromatography for the quantitative profiling of the enzyme-catalyzed hydrolysis of xylo-oligosaccharides

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### **4.1 Key points**

In Paper 2, the xylanase activity was evaluated by the quantification of released xylo-oligosaccharides by HPAEC using commercial xylo-oligosaccharides (DP 2-6). However, the quantification of larger xylo-oligosaccharides was not possible by HPAEC, since xylo-oligosaccharides with higher DP than 6 were not commercially available. The question is whether the quantification of the released xylo-oligosaccharides (DP 2-6) is sufficient for a thorough evaluation of the xylanase activity. No information about the depolymerization of the substrate and the release of larger products upon xylanase treatment could be obtained by HPAEC. In order to at least obtain a qualitative evaluation of the hydrolysis of (arabino)xylan and the products generated during the hydrolysis, HPSEC analysis method was assessed. Where HPAEC typically is used for measuring monosaccharides and smaller oligosaccharides, HPSEC can be used for analysis of the molecular weight distribution of oligo- and polysaccharides in solution (Sørensen et al., 2003).

In HPSEC, the column consists of a hollow tube tightly packed with porous polymer beads designed to have pores of different sizes (Figure 9.A). As the solution travels down the column, smaller particles enter into the pores but larger particles cannot enter as many pores. The larger the particles, the less overall volume to traverse over the length of the column and therefore, these large particles elute faster than smaller particles (Figure 9.B and C).

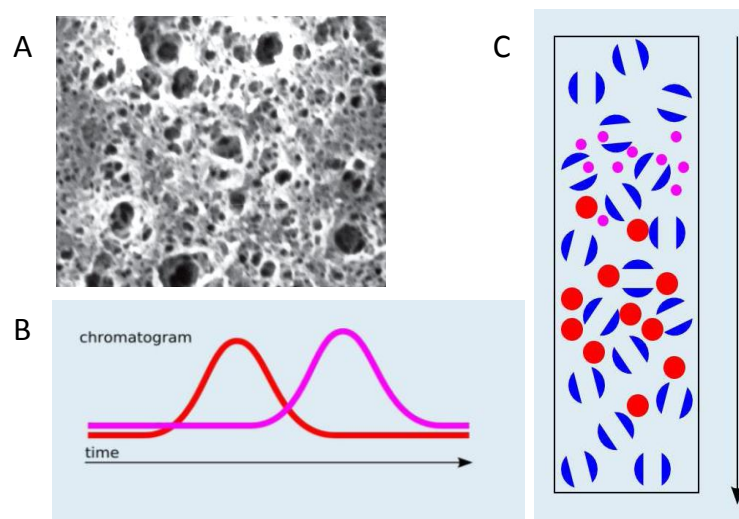


Figure 9. A: Porous polymer beads (Vianna-Soaret et al., 2005), B: Schematic presentation of a size exclusion column. Blue: Porous polymer beads, Red: Large particles, Pink: Small particles; C: Schematic presentation of the detection of size separation by size exclusion chromatography. Red: Large particles, Pink: Small particles. (<http://upload.wikimedia.org/wikipedia/commons/2/2a/SizeExChrom.png>).

However, the disadvantage of this method is that there are no distinct peaks each representing a specific polymer like the HPAEC method. Furthermore, it is a rather imprecise method and comparison can only be made by overlaying profiles. Last, no quantification has been done with method.

The purpose of this article was first to evaluate the influence of specific parameters such as type and concentration of the mobile phase on the chromatographic profile, since apparently no common HPSEC method existed. Furthermore, we particularly wanted to assess whether HPSEC like HPAEC could be used as a quantitative method. By combining the HPAEC and HPSEC methods, a more complete analytical profiling of the progress of the xylanolytic hydrolysis of different xylan substrates could be done.

## 4.2 Conclusion

Paper 3 established that the composition of the mobile phase had a large influence on the HPSEC chromatogram and it was also confirmed that different methods had been used in different research reports to elucidate the xylan depolymerization. The results established that the interference from the sample solution can be reduced to an acceptable minimum by appropriate selection of the mobile phase. The method allowed for a quantification of the xylanolytic activity concerning the capability of catalyzing the depolymerization of the substrate and the following release of smaller and larger xylo-oligosaccharides. Therefore, the hypothesis about the possibility of exploiting HPSEC to obtain a quantitative, or at least semi-quantitative, insight into the evolution of the xylo-oligosaccharides during xylanase treatment of xylan was confirmed.

## Size Exclusion Chromatography for the Quantitative Profiling of the Enzyme-Catalyzed Hydrolysis of Xylo-oligosaccharides

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High-performance size exclusion chromatography (HPSEC) is a widely used method for the qualitative profiling of oligosaccharide mixtures, including, for example, enzymatic hydrolysates of plant biomass materials. A novel method employing HPSEC for the quantitative analytical profiling of the progress of enzymatic hydrolysis of different xylan substrates was developed. The method relies on dividing the HPSEC elution profiles into fixed time intervals and utilizing the linear refractive index response (area under the curve) of defined standard compounds. To obtain optimal HPSEC profiles, the method was designed using 0.1 M CH<sub>3</sub>COONa both in the mobile phase and as the sample solution matrix, after systematic evaluation of the influence of the mobile phase, including the type, ionic strength, and pH, on the refractive index detector response. A time study of the enzyme-catalyzed hydrolysis of birchwood xylan and wheat bran by a *Bacillus subtilis* XynA xylanase (GH 11) was used as an example to demonstrate the workability of the HPSEC method for obtaining progress curves describing the evolution in the product profile during enzyme catalysis.

**KEYWORDS:** Size exclusion; quantitative profiling; xylanase; xylo-oligosaccharides

### INTRODUCTION

Various xylans are currently under intensive study as carbohydrate sources for fermentation to biofuels or as sources of novel health-promoting food ingredients (1–6). Xylan basically consists of a backbone of  $\beta$ -1,4-linked D-xylopyranosyl residues that may be substituted to various degrees with arabinofuranosyl, glucuronate, or acetyl groups. The hydrolysis of the xylan backbone to a mixture of xylo-oligosaccharides can be catalyzed by endo-1,4- $\beta$ -xylanases (xylanases, EC 3.2.1.8) (7). With respect to investigation of the action pattern of endo-1,4- $\beta$ -xylanases and in relation to the production and evaluation of potentially bioactive, prebiotic xylo-oligosaccharides, it is crucial to quantitatively assess the molecular size profile of the oligosaccharides. High-performance size exclusion chromatography (HPSEC) has been employed by several authors for analyzing the molecular weight distribution of different plant oligo- and polysaccharides in solution (5) and to separate polydisperse materials into discrete molecular weight distributions (8). HPSEC is based on permeation of a solute through a column packed with inert porous particles (9). The separation principle relies on the differentiation in the time of permeation of the polysaccharides into and out of the pores of the column packing, which is related to their molecular size. The elution of the polysaccharide is then determined by the time it takes for the polysaccharides to travel through the pores (10). To calculate the molecular weight of the polysaccharides, calibration curves can be constructed by measuring the retention volumes (or retention times) of synthetic polymer standards such as pullulan or polyethylene glycol molecules having a known narrow molar mass distribution (11).

As seen in **Table 1**, HPSEC analysis of the molecular weight distribution of xylan-containing substrates has been used over a broad range of mobile phases at different concentrations and pH values. Sodium chloride, sodium acetate, and sodium nitrate appear to be the most commonly used mobile phases in this application. Furthermore, the sample solutions also differ with respect to type, molar concentration, and pH. Some use the same mobile phase and sample solution, whereas others just inject from any sample matrix. Also seen in **Table 1** is that different detectors have been employed for HPSEC analysis of xylo-oligosaccharide profiles, but differential refractometry detectors are the most widely used (31). Their operation is based on the continuous measurement of the difference in the refractive indices (RI) of the pure mobile phase and the mobile phase containing the substance (32). Generally, the area obtained underneath a RI curve represents the amount of sugars present (22).

When the methods employed are evaluated in detail (**Table 1**), it appears that no common HPSEC method exists and no quantification has been used for xylo-oligosaccharide profiling by HPSEC. This makes it difficult to compare the results obtained by different groups. Moreover, the question is whether the methodology employed has any significance on the results and whether it is possible to use the HPSEC profiles for quantitative purpose. The objectives of the work reported in the present paper were therefore (1) to evaluate the influence of specific parameters such as type and concentration of the mobile phase on the chromatographic profile from HPSEC and (2) to evaluate the possibilities for using HPSEC as a quantitative method to assess xylo-oligosaccharide profiles. As an example, we focused on analyzing the enzyme-catalyzed hydrolysis of different xylan substrates by a wild type *Bacillus subtilis* XynA xylanase. This

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**Table 1.** Various HPSEC Methods That Have Been Used for Assessment of the Molecular Weight of Various Xylan-Containing Substrates<sup>a</sup>

substrate	mobile phase	concn (M)	sample solution	concn (M)	detector	ref
AX (flour)	NaCl	0.05	NaCl	0.05	RI	12
WEAX, WUAX, xylan	NaCl	0.05	CH <sub>3</sub> COONa, pH 6	0.025	RI	13
dough and batter extract	NaCl	0.05	water		RI	14
WEAX and WUAX	NaCl	0.05	CH <sub>3</sub> COONa, pH 4.6	ns	RI	15
extracts of dough and bread	NaCl	0.05	water		RI	16
AX (rye)	NaCl	0.05	ns	ns	UV	17
WEAX (wheat and rye flour and dough)	NaCl	0.1	NaCl	0.1	RI, UV	18
SAX	CH <sub>3</sub> COONa, pH 3	0.4	water		RI	19
water-unextractable cell wall material	CH <sub>3</sub> COONa, pH 3	0.4	water		RI	20
WEAX	CH <sub>3</sub> COONa	0.4	water		RI	5
AX (wheat)	CH <sub>3</sub> COONa, pH 5	0.05	CH <sub>3</sub> COONa, pH 5	0.05	RI	21
liquors after pretreatment of straw	NaNO <sub>3</sub>	0.2	water		RI	22
AX (rye bran)	NaNO <sub>3</sub>	0.1	water		RI, UV, MALLS	23
water-extractable pentosans	NaNO <sub>3</sub>	0.1	water		RI	24
WEAX (wheat)	NaNO <sub>3</sub>	0.05	ns	ns	RI, UV, MALLS	25
maize bran	NaNO <sub>3</sub>	0.05	water		RI, UV, MALLS	26
water-extractable cell wall polysaccharides	NaNO <sub>3</sub> + 0.02% NaN <sub>3</sub>	0.1	NaNO <sub>3</sub> + 0.02% NaN <sub>3</sub>	0.1	RI, MALLS	27
WEAX (wheat flour)	NH <sub>4</sub> HCO <sub>3</sub>	0.002	NH <sub>4</sub> HCO <sub>3</sub>	0.002	RI	28
cell wall fractions (rye grain)	NaNO <sub>3</sub>	0.05	NaNO <sub>3</sub>	0.05	RI, MALLS	29
WEAX	NaN <sub>3</sub>	0.02%	water		ns	4
WEAX and WUAX	water		NaHPO <sub>4</sub> –citric acid, pH 5	0.05	ns	30

<sup>a</sup> AX, arabinoxylan; WEAX, water-extractable arabinoxylan; WUAX, water-unextractable arabinoxylan; SAX, solubilized arabinoxylan; RI, refractive index; MALLS, multiangle laser light scattering; ns, not specified.

particular enzyme has been widely used to study the hydrolysis of arabinoxylan and as a template for evaluating the influence of wheat xylanase inhibitors on xylanase activity (33). The hypothesis was that it was possible to exploit the HPSEC profiles to obtain a quantitative, or at least semiquantitative, insight into the evolution of the xylo-oligosaccharides during endoxylanase treatment of xylan and, in this way, use HPSEC to quantitatively assess different xylo-oligosaccharide product profiles.

## MATERIALS AND METHODS

**Chemicals and Enzymes.** Pullulans with the following average molecular masses,  $0.13 \times 10^4$ ,  $0.6 \times 10^4$ ,  $1 \times 10^4$ , and  $40 \times 10^4$  g mol<sup>-1</sup>, were from Fluka Chemie AG (Buchs, Switzerland) and D-(+)-xylose was from Merck (Darmstadt, Germany). Xylohexaose was obtained from Megazyme (Bray, Ireland).

For the destarching of wheat bran,  $\alpha$ -amylase (Termamyl SC, Novozymes A/S (Bagsværd, Denmark)) and amyloglucosidase (Spirizyme Plus FG, Novozymes A/S) were used.

The *B. subtilis* xylanase (BsX) was obtained from Danisco A/S (Brabrand, Denmark). BsX carries a Swiss Prot entry P18429, XynA\_BACSU and has been classified as a glycosyl hydrolase family 11 xylanase according to sequence homology (34). The xylanase was purified from a Grindamyl H 640 enzyme preparation and was purified according to the method of Sørensen and Sibbesen (33). BsX had a molecular mass of 20 kDa, pH optimum at 6, and temperature optimum at 50 °C.

All other reagents used were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium), unless specified otherwise.

**Substrates and Substrate Pretreatment.** Water-unextractable arabinoxylans (WUAX) (arabinose/xylose (A/X) = 0.61) from wheat were obtained from Megazyme. Birchwood xylan was purchased from Sigma-Aldrich and was solubilized with ethanol as recommended in the product sheet. Wheat bran was a commercial product from Natur Drogeriet A/S (Hørning, Denmark). The wheat bran was destarched via sequential treatment with  $\alpha$ -amylase (Termamyl SC) (95 °C, pH 6, 60 min) and amyloglucosidase (Spirizyme Plus FG) (60 °C, pH 4.5, 30 min) followed by ethanol precipitation. Acid hydrolysis (0.4 M HCl, 2 h, 100 °C) (5) of birchwood xylan and wheat bran showed that the monosaccharide composition of the birchwood xylan was 1.0 wt % galactose, 1.9 wt % glucose, and 97.1 wt % xylose and that of the destarched wheat bran was 3 wt % galactose, 10 wt % glucose, 32 wt % arabinose, and 55 wt % xylose (measured by HPAEC according to the method of Arnous and Meyer (35)).

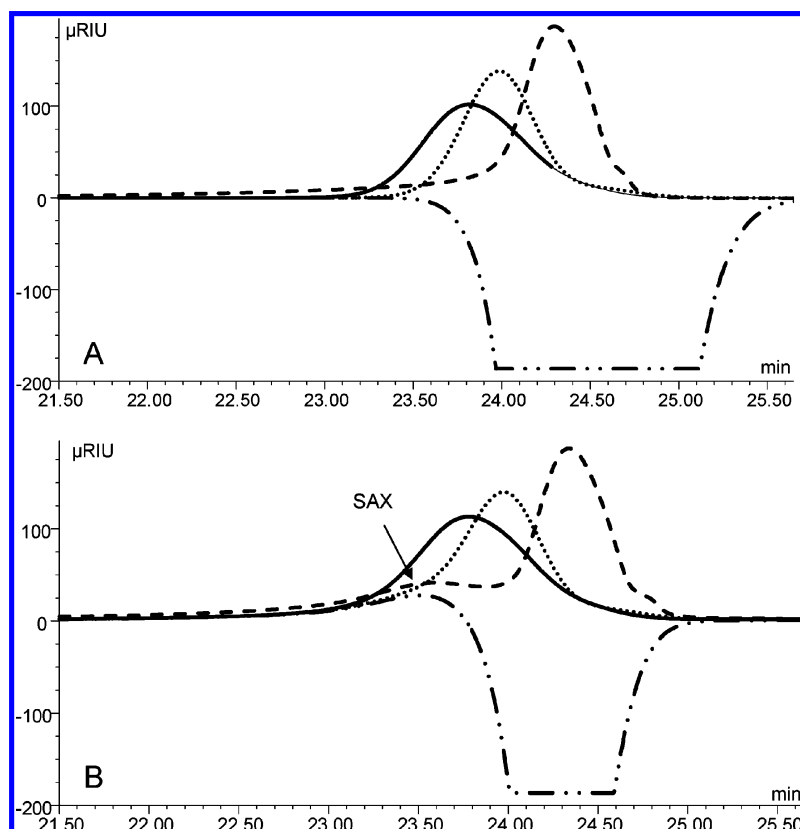
**Enzymatic Hydrolysis.** Enzyme-catalyzed hydrolysis of WUAX, birchwood xylan, and wheat bran by BsX was assessed according to the following general procedure: The substrate (1 wt %) was dissolved in 0.1 M sodium acetate, pH 6, with 0.02 wt % sodium azide. The substrate was preincubated for 10 min at 40 °C under continuous stirring before a suitably diluted enzyme solution, to give a final reaction concentration of 0.12  $\mu$ mol of enzyme g<sup>-1</sup> dry matter substrate, was added. For birchwood xylan and wheat bran, samples were withdrawn after 0, 4, 8, 15, 30, 60, 120, and 240 min and for WUAX only after 24 h. The enzymatic hydrolysis was stopped immediately by heating the samples at 100 °C for 10 min. After centrifugation (10000g, 10 min), the hydrolysates were analyzed for molecular weight distribution by HPSEC.

**High-Performance Size Exclusion Chromatography.** HPSEC was carried out using a system consisting of a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex Corp., Sunnyvale, CA). Samples were separated on a Shodex SB-806 HQ GPC column (300  $\times$  8 mm) with a Shodex SB-G guard column (50  $\times$  6 mm) from Showa Denko K.K. (Tokyo, Japan). Different mobile phases were used with a flow rate of 0.5 mL min<sup>-1</sup>, and the injection volume was 0.05 mL. All chromatographic experiments were conducted at 40 °C in a thermostated heater, and the columns were equilibrated overnight with various mobile phases prior to the start of any experiment.

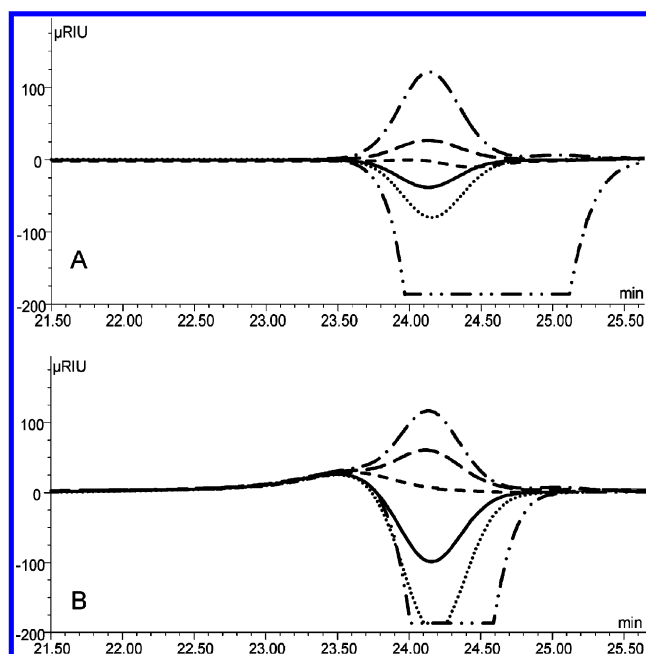
Molecular mass markers (0.5 mg mL<sup>-1</sup>) were pullulans with molecular masses of  $0.13 \times 10^4$ ,  $0.6 \times 10^4$ ,  $1 \times 10^4$ , and  $40 \times 10^4$  g mol<sup>-1</sup>, xylohexaose, and xylose. Furthermore, dextran with a molecular mass of  $11 \times 10^4$  g mol<sup>-1</sup> was used.

## RESULTS

**Effect of the Mobile Phase.** *Type of Mobile Phase.* **Figure 1** displays the chromatograms of the sample solution (0.1 M CH<sub>3</sub>COONa, pH 6) (**Figure 1A**) and hydrolysates of arabinoxylan (solubilized arabinoxylan, SAX) in sample solution (**Figure 1B**). When only the sample solution was loaded on the HPSEC system, chromatographic peaks still appeared that corresponded to the salt ions in the sample solution (**Figure 1A**). An eluent of 0.4 M CH<sub>3</sub>COONa, pH 3, gave a relatively large negative peak, whereas the other three eluents employed gave a positive peak, but with various elution times and shapes. Elution with water and 0.4 M CH<sub>3</sub>COONa, pH 3, resulted in the appearance of a peak in the chromatogram after approximately 23.5 min corresponding to SAX (**Figure 1B**). However, when a mixture of 0.05 M NaCl and CH<sub>3</sub>COONH<sub>4</sub> was used as the

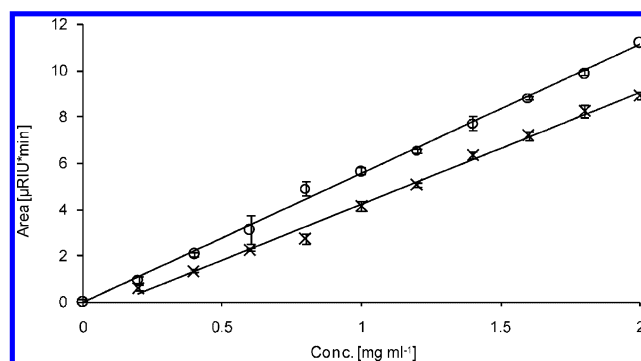


**Figure 1.** Size exclusion chromatograms of (A) sample solution (0.1 M  $\text{CH}_3\text{COONa}$ , pH 6) and (B) hydrolysate of arabinoxylan (SAX) in sample solution. The samples are eluted with the following different types of mobile phases: water (---), 0.05 M NaCl (—), 0.05 M  $\text{CH}_3\text{COONH}_4$  (···), and 0.4 M  $\text{CH}_3\text{COONa}$ , pH 3 (— · —).



**Figure 2.** Effect of the ionic strength of the mobile phase ( $\text{CH}_3\text{COONa}$ , pH 6): chromatographic profiles of (A) sample solution (0.1 M  $\text{CH}_3\text{COONa}$ , pH 6) and (B) hydrolysate of arabinoxylan (SAX). The samples were eluted with the following concentrations of  $\text{CH}_3\text{COONa}$  as the mobile phase: 0.05 M (— · —), 0.075 M (— — —), 0.1 M (---), 0.15 M (—), 0.2 M (· · ·), and 0.4 M (— · · —).

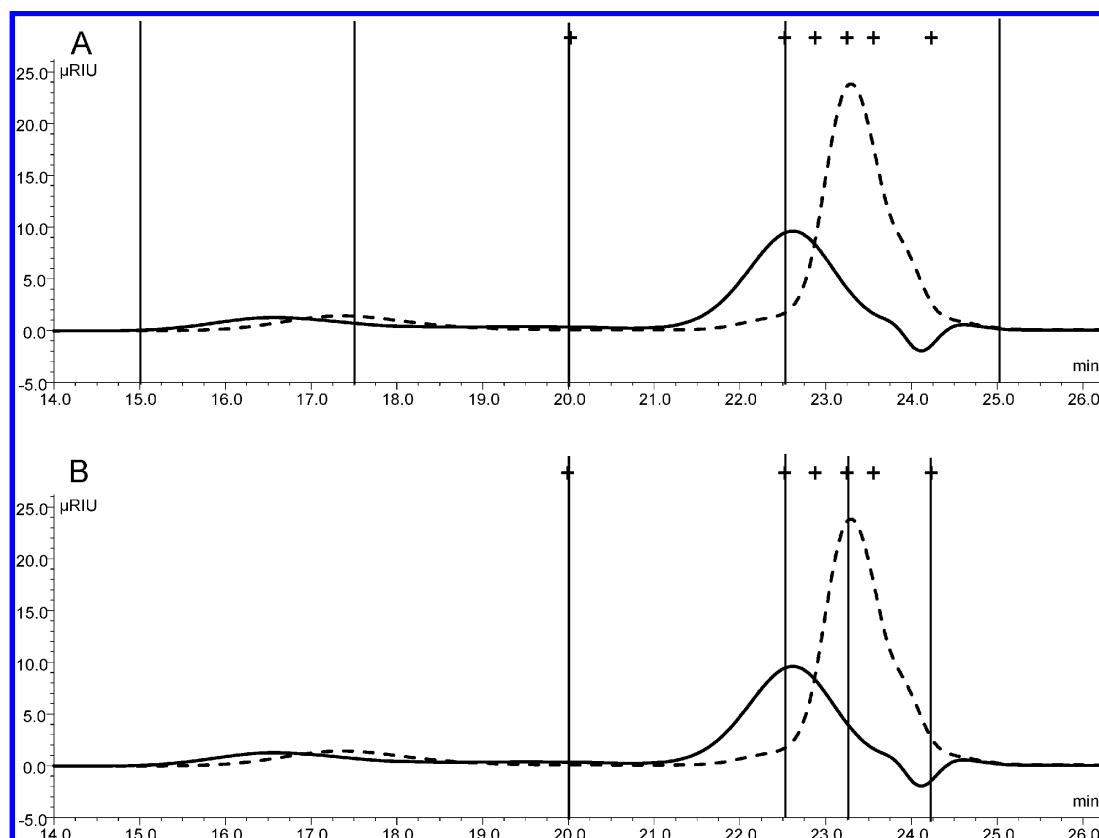
eluent, the peak from SAX was shielded by the chromatographic peak from the sample solution.



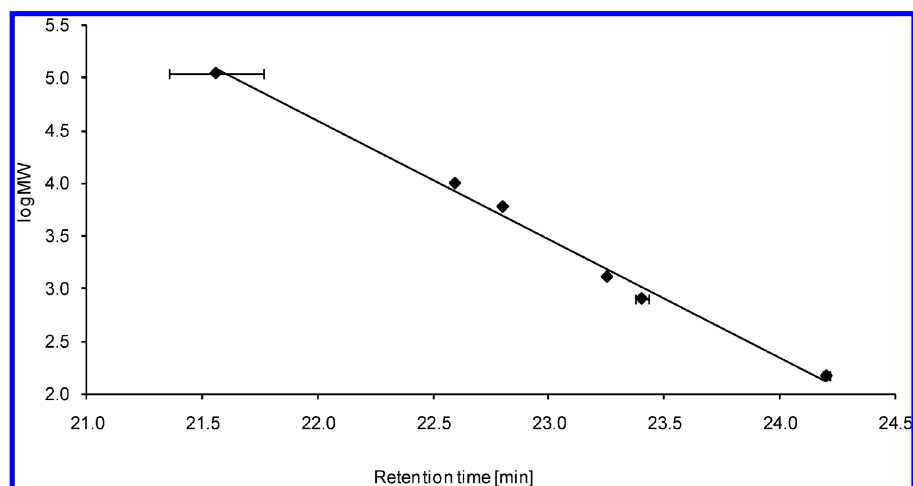
**Figure 3.** Standard curve (0.1–1  $\text{mg mL}^{-1}$ ) for pullulan ( $1.0 \times 10^4 \text{ g mol}^{-1}$ ) ( $\odot$ ) and xylohexaose ( $0.081 \times 10^4 \text{ g mol}^{-1}$ ) ( $\times$ ). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation.

**Concentration of the Mobile Phase.** To assess the effect of the ionic strength of the mobile phases on the chromatographic profiles, the influence of different concentrations (0.05–0.4 M) of one type of mobile phase ( $\text{CH}_3\text{COONa}$ , pH 6) on the chromatograms was studied (Figure 2). In all of the profiles obtained, a relatively large peak appeared after approximately 24 min, which corresponded to the sample solution. The polarity of this peak could be correlated to the ionic strength of the mobile phase: Mobile phases with low ionic strength (0.05–0.075 M) resulted in a positive peak and those with high ionic strength (0.1–0.4 M) in negative peaks. When SAX (Figure 2B) was eluted with different concentrations of the mobile phase, a second peak appeared at  $t = 23.5$  min corresponding to the eluted molecules. When using 0.1 M  $\text{CH}_3\text{COONa}$ , pH 6, as mobile phase, it was





**Figure 4.** Two strategies for calculating the area of chromatographic peaks: (A) calculation of area based on fixed time intervals; (B) calculation of area based on time intervals made from the retention time of specific standards. Molecular mass markers (+) from left to right are  $40 \times 10^4$ ,  $1 \times 10^4$ ,  $0.6 \times 10^4$ ,  $0.13 \times 10^4$ , xylohexaose, and xylose. The vertical lines in both panels represent time intervals.



**Figure 5.** HPSEC calibration plot using the following molecular mass markers ( $0.5 \text{ mg mL}^{-1}$ ): pullulans with molecular masses of  $0.13 \times 10^4$ ,  $0.6 \times 10^4$ ,  $1 \times 10^4$ , and  $40 \times 10^4 \text{ g mol}^{-1}$ , dextran ( $11 \times 10^4 \text{ g mol}^{-1}$ ), xylohexaose ( $0.081 \times 10^4 \text{ g mol}^{-1}$ ), and xylose ( $0.015 \times 10^4 \text{ g mol}^{-1}$ ). Values represent the mean of three independent measurements and are shown  $\pm 1$  horizontal standard deviation.

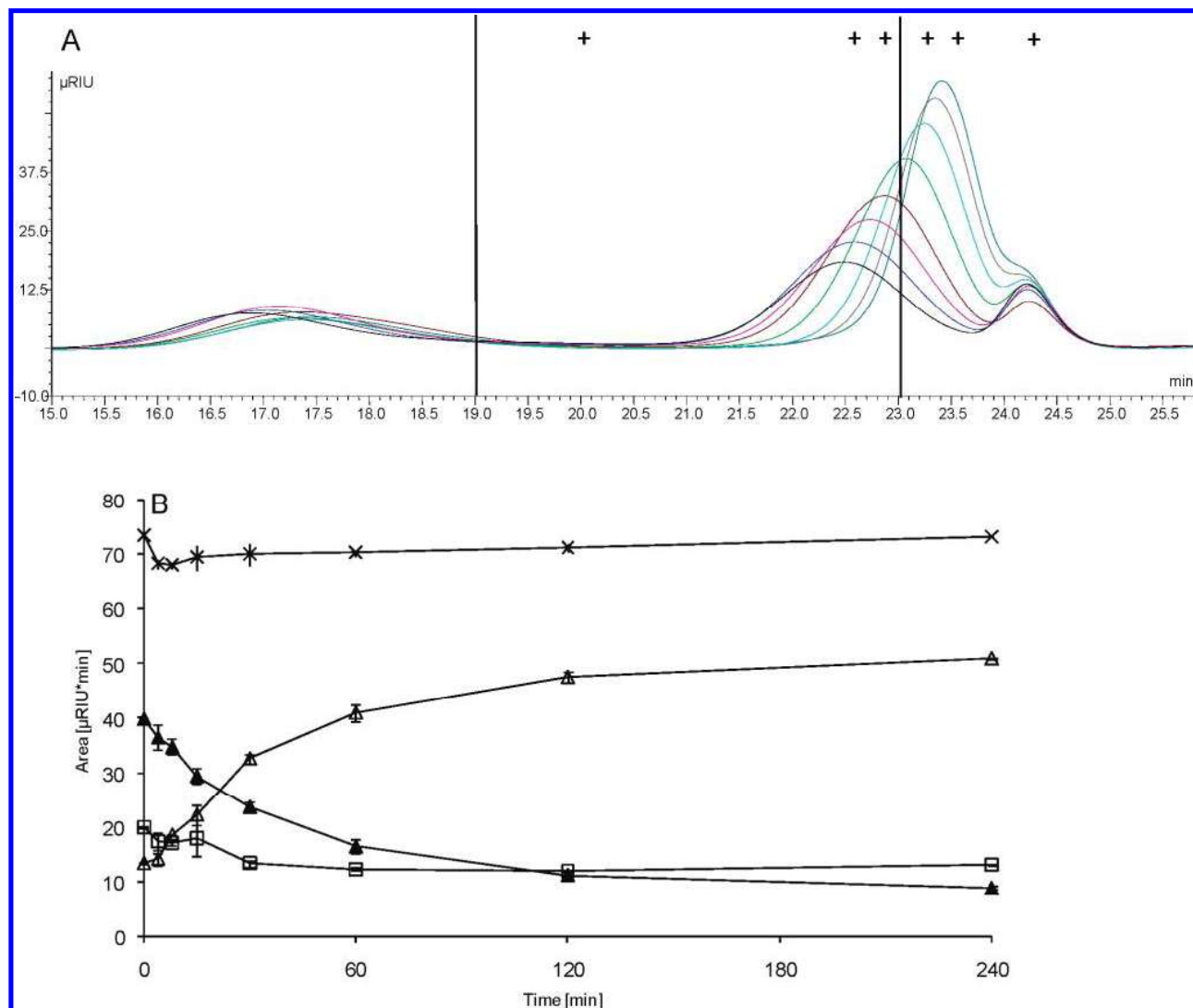
possible to almost eliminate the chromatographic peak from the sample solution. At this concentration the interference from the sample solution peak was so small that it was acceptable. On the basis of the HPSEC profile the molecular mass of SAX was determined to be  $650 \text{ g mol}^{-1}$ , equivalent to an average degree of polymerization (DP) in the SAX of  $\sim 4.8$ .

The pH of the mobile phase was decreased to 3 to assess its effect on the chromatographic profiles. An extra peak at 24.3 min occurred with both the sample solution and SAX (data not shown). This indicated that when the pH was lowered, the ionic

strength of the mobile phase was increased, and therefore a second peak appeared.

**Quantification.** To verify that the area beneath a refractive index curve profile could be correlated to the concentration, a standard curve was measured for pullulan ( $1.0 \times 10^4 \text{ g mol}^{-1}$ ) and xylohexaose ( $0.081 \times 10^4 \text{ g mol}^{-1}$ ), both at concentrations of  $0.2\text{--}2 \text{ mg mL}^{-1}$ .  $R^2$  values of 0.998 for pullulan and 0.994 for xylohexaose were obtained, indicating a linear correlation between the area and the concentration (Figure 3). The values for the area of xylohexaose at various concentrations were in general





**Figure 6.** Catalyzed hydrolysis of birchwood xylan by BsX. **(A)** HPSEC chromatographic profile after 0 min (black), 4 min (dark blue), 8 min (pink), 15 min (brown), 30 min (green), 60 min (light blue), 120 min (gray), and 240 min (gray blue). Molecular mass markers (+) from left to right are  $40 \times 10^4$ ,  $11 \times 10^4$ ,  $1 \times 10^4$ ,  $0.6 \times 10^4$ ,  $0.13 \times 10^4$ , xylohexaose, and xylose. **(B)** Progression curve of the area in the following time intervals: 15–19 min ( $\square$ ), 19–23 min ( $\blacktriangle$ ), 23–27 min ( $\triangle$ ), total area 15–27 min ( $\times$ ). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation. The vertical lines represent time intervals.

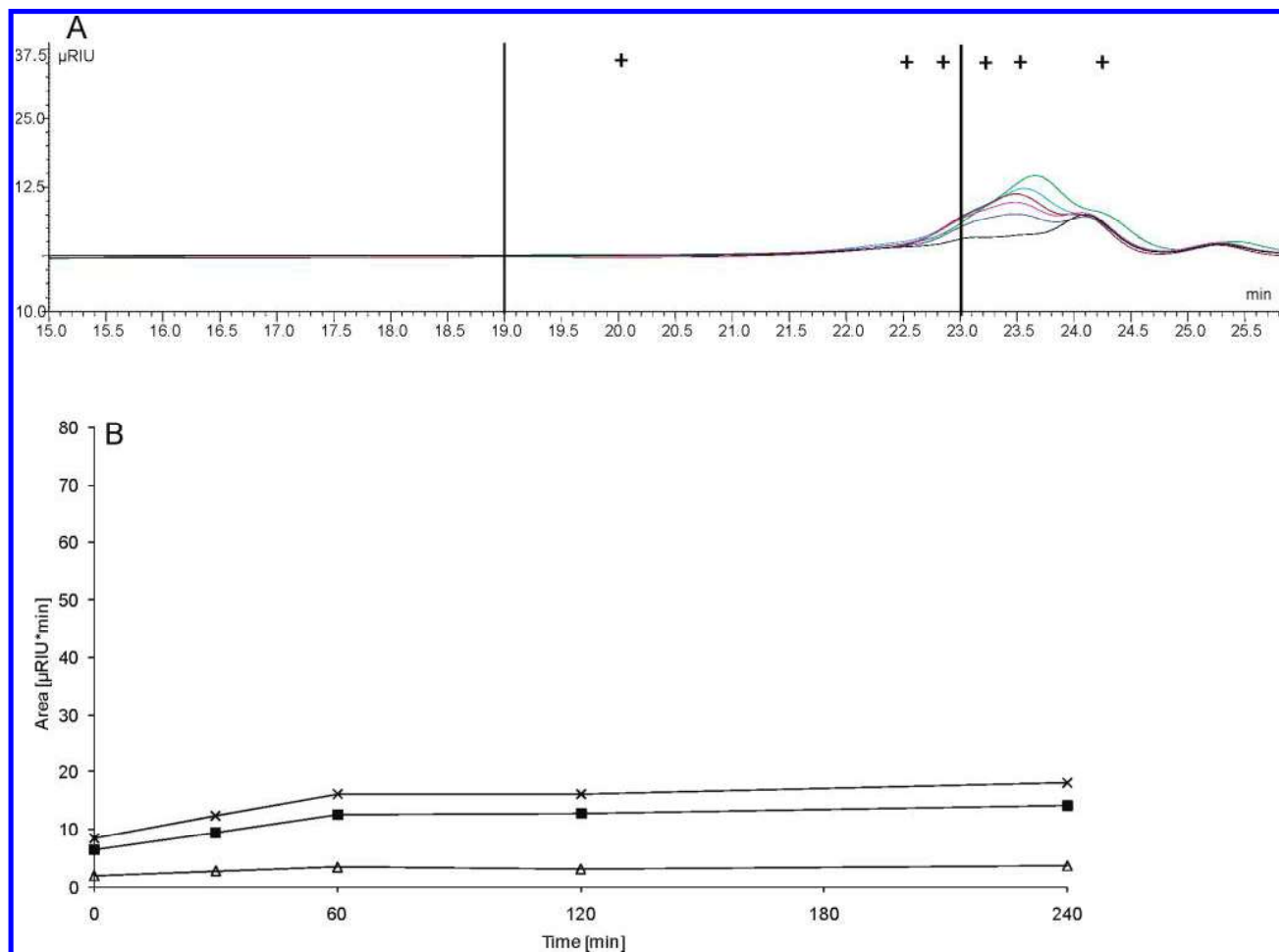
slightly lower than those obtained for pullulan, and therefore the tendency line does not go through the origin. Both standards were solubilized in water, and a relatively large negative peak corresponding to the water interfered with the chromatographic peak of xylohexaose, making the xylohexaose peak slightly smaller (data not shown). The negative peak appeared because the ionic strength of the sample was lower than that of the mobile phase. This was equivalent to when the sample solution (0.1 M  $\text{CH}_3\text{COONa}$ , pH 6) was eluted with  $\text{CH}_3\text{COONa}$ , pH 6, mobile phases at 0.15, 0.2, and 0.4 M, resulting in a negative peak (Figure 2A).

The slopes of both standard curves appeared to be similar:  $5.5 \mu\text{RIU min mL mg}^{-1}$  for pullulan and  $4.8 \mu\text{RIU min mL mg}^{-1}$  for xylohexaose. However, a comparison of the 95% confidence intervals showed that there was a significant difference between the two slopes. Hence, for further quantification, pullulan was used, because the molecular mass was more equivalent to the assessed hydrolysis of birchwood xylan and wheat bran.

In principle, two different strategies may be applied when the area of the chromatographic profiles is calculated to quantify molecules with a specific molecular weight distribution. First, the

profile could be divided into fixed time intervals that are not directly correlated to the retention time of specific standards (Figure 4A). The area of the profile could then be calculated for each time interval. A second strategy could be to use time intervals based on the retention time of specific standards (Figure 4B). However, most likely, the time intervals would then have different lengths and therefore have to be standardized by dividing the calculated area by the time interval. This would imply that much information would be lost. We therefore continued with fixed time intervals to make a quantification of the different molecular weight distributions.

**Calibration with Molecular Weight Standards.** The apparent molecular mass of a sample was determined by comparison of the chromatographic behavior of the sample to that of standards with known molecular weights. Pullulan, dextran, xylohexaose, and xylose standards ranging in molecular mass from  $0.015 \times 10^4$  to  $11 \times 10^4 \text{ g mol}^{-1}$  were used for the calibration (Figure 5). An  $R^2$  value of 0.993 was obtained, indicating a linear relationship between the retention time and the logarithm to the molecular weight, at least in the range of the used standards.



**Figure 7.** Catalyzed hydrolysis of wheat bran by BsX. **(A)** HPSEC chromatographic profile after 0 min (black), 30 min (dark blue), 60 min (pink), 120 min (brown), 240 min (light blue), and 24 h (green). Molecular weight markers (+) from left to right are  $40 \times 10^4$ ,  $11 \times 10^4$ ,  $1 \times 10^4$ ,  $0.6 \times 10^4$ ,  $0.13 \times 10^4$ , xylohexaose, and xylose. **(B)** Progression curve of the area in the following time intervals: 19–23 min ( $\Delta$ ), 23–27 min ( $\blacksquare$ ), total area 15–27 min ( $\times$ ). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation. The vertical lines represent time intervals.

**Enzymatic Hydrolysis.** The strategy with fixed time intervals was applied on a time study of the catalyzed hydrolysis of birchwood xylan by BsX (Figure 6A). Because this substrate was water-soluble, the initial profile of birchwood xylan after 0 h of incubation showed its molecular weight distribution (black line, Figure 6A). The initial chromatographic profile was composed of three peaks, where the first peak appeared after 16.8 min. Because this was earlier than the elution of the  $40 \times 10^4 \text{ g mol}^{-1}$  molecular mass standard, these fragments had a molecular mass larger than  $40 \times 10^4 \text{ g mol}^{-1}$ . The birchwood xylan also contained smaller fragments, eluting at 22.5 min, and the molecular mass of these fragments was estimated to be  $1.1 \times 10^4 \text{ g mol}^{-1}$  using the calibration curve (Figure 5). Because a third peak appeared at the same time as the standard xylose (24.2 min), some xylose was also present in the substrate solution. During the incubation period, the molecular mass of the large and small fragments decreased as a result of the xylanase activity, where the molecular mass of the small fragments reached approximately  $0.09 \times 10^4 \text{ g mol}^{-1}$  after 4 h. The apparent molecular mass of the large fragments decreased only relatively little during the incubation period and was still larger than  $40 \times 10^4 \text{ g mol}^{-1}$ . This relatively small change might be explained by the HPSEC setup where only one HPSEC column, being able to separate low molecular mass fragments and not molecules larger than  $40 \times 10^4 \text{ g mol}^{-1}$ , was used.

The HPSEC profiles were divided into three time intervals that resulted in an equal time division of 4 min, and the area

underneath the curve in each interval was calculated (Figure 6B). The selection of the number of intervals was made as a compromise between obtaining detailed information and having a manageable overview. The calibration curve in Figure 5 was used to convert retention times into molecular weights and the standard curve for pullulan in Figure 3 for converting areas to concentrations.

For the time interval 15–19 min, which represented molecules larger than  $40 \times 10^4 \text{ g mol}^{-1}$ , the area decreased only a little and mainly during the first 30 min of enzymatic treatment (Figure 6B). On the basis of the areas in this time interval, the concentration of these relatively large fragments was  $3.59 \text{ mg mL}^{-1}$  at 0 min and  $2.37 \text{ mg mL}^{-1}$  after 240 min. For the time interval 19–23 min representing fragments with a molecular mass between  $0.29 \times 10^4$  and  $40 \times 10^4 \text{ g mol}^{-1}$ , the area decreased mainly during the first 120 min. The concentration of these relatively smaller fragments prior to the enzymatic hydrolysis was  $7.15 \text{ mg mL}^{-1}$  and, after 240 min,  $1.62 \text{ mg mL}^{-1}$ . In contrast to the other two intervals, the area in the period 23–27 min representing fragments smaller than  $0.29 \times 10^4 \text{ g mol}^{-1}$  increased during the whole incubation period, however, leveling off after 120 min. At the beginning of the incubation period, the concentration was  $2.41 \text{ mg mL}^{-1}$ , and it increased to  $9.13 \text{ mg mL}^{-1}$  after 240 min.

The rate of attack of the xylanase could tentatively be evaluated by calculating the initial rate of decrease or increase in each time interval of the area curves for the first 15 min (Figure 6B). When the slopes were compared, the slope of the 15–19 min curve

(0.14  $\mu$ RIU) was smaller than the one of the time interval 19–23 min (0.70  $\mu$ RIU). The rate of decrease for the 19–23 min time interval was similar to the rate of increase in the time interval 23–27 min (0.59  $\mu$ RIU). This indicated that the concentration of the smaller fragments ( $0.29 \times 10^4$ – $40 \times 10^4$  g mol<sup>-1</sup>) decreased more quickly than that of the large fragments ( $4 \times 10^5$  g mol<sup>-1</sup>). It could therefore be inferred that the xylanase appeared to have a higher affinity for the smaller fragments, but the rate difference might also be a result of the differences in the concentration of the two different molecular size (substrate) profile groups.

When wheat bran was hydrolyzed by BsX, no molecules above  $40 \times 10^4$  g mol<sup>-1</sup> were measured (Figure 7A). Because wheat bran was insoluble and therefore was removed by centrifugation after the enzymatic hydrolysis, the HPSEC profiles represent only the soluble fragments released from wheat bran by the BsX action. On the basis of Figure 7A, the molecular mass of the released fragments was smaller than  $0.55 \times 10^4$  g mol<sup>-1</sup>. During the incubation period the molecular mass of the fragments decreased to approximately 470 g mol<sup>-1</sup> after 24 h of hydrolysis. This indicated that the released fragments apparently were the preferred substrate for the xylanase.

As in Figure 6A, the profile in Figure 7A was divided into the same fixed time intervals to be able to compare results from both reactions. However, only the intervals 19–23 and 23–27 min were used, because no fragments eluted earlier. As seen in Figure 7B, the area increased in both intervals mainly for the first hour and then only increased slightly. The initial concentration of the fragments with a molecular mass between  $0.29 \times 10^4$  and  $40 \times 10^4$  g mol<sup>-1</sup> (eluting in the interval 19–23 min) was 0.36 mg mL<sup>-1</sup> and increased to 0.66 mg mL<sup>-1</sup> after 240 min of incubation. For fragments smaller than  $0.29 \times 10^4$  g mol<sup>-1</sup>, the concentration increased from 1.17 mg mL<sup>-1</sup> after 0 min to 2.55 mg mL<sup>-1</sup> after 240 min of incubation. The initial rate of increase in the area in the time interval 23–27 was relatively larger (0.101  $\mu$ RIU) than that in the 19–23 min period (0.028  $\mu$ RIU). This, together with the larger area, again reflected the apparent preference of the xylanase for the smaller, or at least the soluble, fragments.

## DISCUSSION

On the basis of Figures 1 and 2, it is seen that the type, concentration, and pH of the mobile phase largely influence the chromatographic peak of the sample solution with respect to size, retention time, and polarity. When SAX was eluted, the peak from the sample solution interfered with the peak of interest, either decreasing or increasing the area and thereby making the concentration estimation less precise. The size and polarity of the sample solution peak was correlated to the ionic strength of the mobile phase, indicating that the RI detector reacted on differences in ionic strength between the sample and the mobile phase. To reduce the chromatographic peak from the sample solution, the ionic strength difference between the sample and the mobile phase had to be minimized. These results correspond with the recommendations from the HPSEC column manufacturer (Showa, Munich, Germany) that samples should be dissolved in the same sample solution as the mobile phase when a differential refractometer is used. However, our literature search illustrated in Table 1 showed that this was only done in a few of the references. Le Coupanec et al. (36) also observed a slight alternation of the retention time in the chromatograms when using NaNO<sub>3</sub> and CH<sub>3</sub>COONH<sub>4</sub> as eluents for HPSEC. This could be due to the influence of the nature of the salt on the matrix pore and the compound size.

Once the interference from the sample solution was minimized, the area of a chromatographic profile could in principle be used for quantification, because there was a linear correlation between

the area and the concentration of specific standards (Figure 3). This linear correlation has been widely used in high-performance anion exchange chromatography (HPAEC) for quantification, because most samples can be separated into distinct peaks when concentrations of specific molecules are estimated on the basis of a standard curve of known standards (35). However, HPSEC has mostly been used for measuring the molecular weight distribution, and therefore one peak could represent molecules with different molecular weights (8). HPSEC has therefore until now mainly been used as a qualitative method, for monitoring the molecular weight distribution.

With this new method, it has now been possible to quantify how much a molecular weight distribution has changed and also to calculate parameters such as standard deviation to tentatively estimate the reproducibility of the experiment and the method. Furthermore, the contribution from the substrate itself to the molecular weight distribution can now be subtracted, so only the enzyme activity can be estimated. This feature is particularly relevant when one is dealing with substrates such as wheat bran, which tend to autohydrolyze; at least, wheat bran autohydrolyzes to a higher degree than, for example, birchwood xylan.

Despite our literature search on HPSEC, we found that only Kabel et al. (22) used a similar semiquantitative method based on division of the HPSEC profiles into retention time intervals. In their work, the HPSEC profile was divided into three retention time intervals of different lengths based on standards representing molecules of high (DP > 25), medium (DP 9–25), and low (DP < 9) molecular weights (22). For the three parts, the percentage of the total area in the HPSEC diagram was calculated. By using this method, the internal molecular weight distribution was depicted after a certain treatment. However, when using this method, it would most likely not be possible to depict if the total area increased, because the area in each interval also would increase. Therefore, an increase or decrease in the total concentration would most likely not be detected.

In the present study, birchwood xylan and wheat bran were enzymatically catalyzed by treatment with the xylanase BsX (Figures 6 and 7). On the basis of the HPSEC profiles, fragments with a molecular mass below  $6 \times 10^3$  g mol<sup>-1</sup> were produced from both substrates, mainly during the first 4 h of incubation. When fragments after 4 h were compared, higher concentrations were released from birchwood xylan than from wheat bran. This corresponds to earlier results when the same enzymatic hydrolysis was analyzed by HPAEC, yielding a total amount of xylo-oligosaccharides (DP 2–6) of 6.5 mM released from birchwood xylan and of 3 mM from wheat bran (37). The observed differences in HPAEC and HPSEC of the two substrates may be related to differences in the complexity of the substrates. Because birchwood xylan was composed of approximately 97% xylose (37) and therefore only had relatively few arabinose substituents, this substrate was more accessible than wheat bran, where lower levels of fragments were produced. This was probably due to the more complex composition, where physical access to the  $\beta$ -1,4 linkages in xylan was restricted by the various substituents on the xylan backbone (38).

In any case, the appropriate selection of the mobile phase composition is important, because it has a large influence on the chromatographic profile of xylo-oligosaccharides. When using the same mobile phase as the sample matrix solution, this study showed that the interference from the sample solution was reduced to an acceptable minimum in the one-column HPSEC separation profile. A new method suitable for quantifying the molecular weight distribution of enzymatic hydrolysis allowed a more precise and detailed quantification of the product. The HPSEC can be used not only as a qualitative method but also as a quantitative method where more information can be gained from

HPSEC chromatograms. It is our belief that the quantitative approach reported here may be applied to other types of carbohydrate substrates and hydrolysates.

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## Chapter 5 Endogenous $\beta$ -D-xylosidase and $\alpha$ -L-arabinofuranosidase activity in flaxseed mucilage

This chapter is extended in the form of Paper 4:

Endogeneous  $\beta$ -D-xylosidase and  $\alpha$ -L-arabinofuranosidase activity in flaxseed mucilage

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### 5.1 Key points

In Paper 1, 2 and 3 commercial substrates like WE-AX, WU-AX and birchwood xylan were used for the enzymatic production of xylo-oligosaccharides. However, if the generated xylo-oligosaccharides were to be tested for a potential prebiotic effect in e.g. mono-bacterial cultures, a minimum of 100 mg is needed (Holck et al., 2011). If commercial substrates were used as source for the generation of xylo-oligosaccharides, then this process would not be economical feasible and therefore, other substrates were considered. Since arabinoxylan was present in relatively high amounts in flaxseed mucilage (FM) and had an interesting substitution pattern, the potential of this substrate as a source for production of xylo-oligosaccharides was investigated.

#### 5.1.1 Structure of arabinoxylan from flax seed mucilage

Flax is a annual plant from the *Linaceae* family and the flaxseed are known as a functional food source related to their content of protein and soluble fiber (Oomah et al., 2001), and for their medicinal properties like anti-inflammatory effect according to the presence of  $\alpha$ -linolenic acid (Clark et al., 1995). Arabinoxylan present in the mucilaginous cells of the seeds, called mucilage after extraction, is much more branched compared to cereals like wheat. The polysaccharide contains L-arabinofuranosyl units at the same C(O)-2 and C(O)-3 positions along the xylan backbone and the total ratio of branched to unbranched residues of isolated arabinoxylan is approximately 1.1:1.0. In wheat this ratio is 2:3 (Sørensen et al., 2006). The doubly substituted  $\beta$ -1,4-linked D-xylopyranosyl units, that have single, L-arabinosyl units attached via ( $\alpha$ -1,2) and ( $\alpha$ -1,3) bonds, constitute almost 80 mol% of the arabinofuranosyl substituted xylose residues in the arabinoxylan backbone (Naran et al. 2008). Moreover, this polysaccharide contains high amount of D-galactose and L-fucose units (Guilloux et al. 2010; Naran et al. 2008).

The tentative structure of arabinoxylan from flax seed mucilage is depicted in Figure 10.

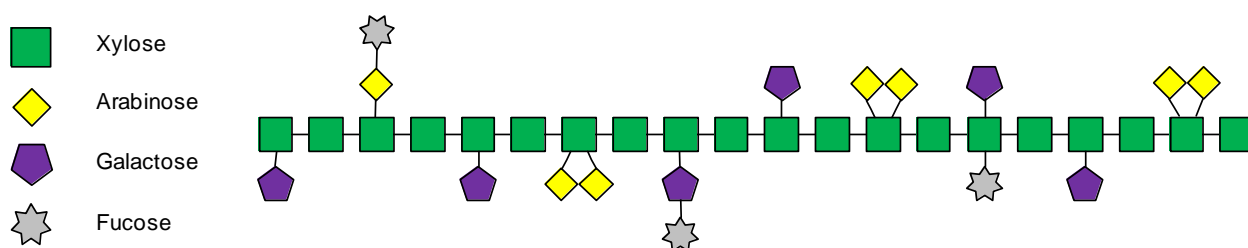


Figure 10. Proposed schematic representation of the flaxseed mucilage arabinoxylan (based on composition results from Guilloux et al. 2010 and Naran et al., 2008). The xylopyranosyl residues in the xylan backbone are  $\beta$ -1,4 linked, whereas the substituents are connected to the backbone of xylan via an  $\alpha$ -1,2- and/or  $\alpha$ -1,3-linkage.

## 5.2 Conclusion

BsX used in this study had only limited solubilising capacity, and was only able to catalyze the depolymerization the xylan backbone to a limited extent. The hypothesis for this work was that flaxseed mucilage could be used as a substrate for enzymatic production of xylo-oligosaccharides by BsX. If this substrate is to be used for the production of xylo-oligosaccharides, enzyme mixtures containing different kinds of enzyme activities will be necessary for the catalyzed solubilisation and/or depolymerization of the flaxseed arabinoxylan. Enzymes catalyzing the releasing the arabinofuranosyl or galactopyranosyl substitutions can probably assist the xylanase, since these enzyme activities leave behind a polysaccharide with longer stretches of unsubstituted xylopyranosyl residues, which in turn can be hydrolyzed by the xylanase.

## Endogeneous $\beta$ -D-xylosidase and $\alpha$ -L-arabinofuranosidase activity in flax seed mucilage

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**Abstract** Flax seed mucilage (FM) contains a mixture of highly doubly substituted arabinoxylan as well as rhamnogalacturonan I with unusual side group substitutions. Treatment of FM with a GH11 *Bacillus subtilis* XynA endo 1,4- $\beta$ -xylanase (BsX) gave limited formation of reducing ends but when BsX and FM were incubated together on different wheat arabinoxylan substrates and birchwood xylan, significant amounts of xylose were released. Moreover, arabinose was released from both water-extractable and water-unextractable wheat arabinoxylan. Since no xylose or arabinose was released by BsX addition alone on these substrates, nor without FM or BsX addition, the results indicate the presence of endogenous  $\beta$ -D-xylosidase and  $\alpha$ -L-arabinofuranosidase activities in FM. FM also exhibited activity on both *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside (*p*NPA) and *p*-nitrophenyl  $\beta$ -D-xylopyranoside (*p*NPX). Based on  $K_M$  values, the FM enzyme activities had a higher affinity for *p*NPX ( $K_M$  2 mM) than for *p*NPA ( $K_M$  20 mM).

**Purpose of work** Endogenous  $\beta$ -D-xylosidase (EC 3.2.1.37) and  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55) activities have been detected and studied in flax seed mucilage (FM).

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**Keywords** Arabinofuranosidase · Arabinoxylan ·  $\beta$ -xylosidase · Flax seed · Mucilage

### Introduction

Seeds from flax (*Linum usitatissimum*) are increasingly used in foods and are recognized to exert potentially positive effects on health due to their high contents of  $\omega$ -3 lipids, antioxidants, and dietary fiber (Weill and Mairesse 2010). When flax seeds are wetted, the epidermal cells of the flax seed coats release large quantities of mucilage that form a gel-like capsule surrounding the seeds (Naran et al. 2008). This flax seed mucilage (FM) contains a mixture of arabinoxylan (AX) and rhamnogalacturonan I polysaccharides having rare side chain substitutions such as single terminal D- and L-galactose and L-fucose residues (Warrand et al. 2005a, b; Naran et al. 2008). In the AX isolated from FM the ratio of branched to unbranched xylose residues is  $\sim$ 1.1:1 and the doubly substituted  $\beta$ -1,4-linked D-xylopyranosyl units, that have single, L-arabinosyl units attached via ( $\alpha$ 1  $\rightarrow$  2) and ( $\alpha$ 1  $\rightarrow$  3) bonds, constitute almost 80 mol% of the arabinofuranosyl substituted xylose residues in the AX backbone (Naran et al. 2008).

Enzyme-catalyzed degradation of cereal-derived AX is currently receiving significant attention both in relation to biofuel processing and prebiotic food ingredients production (Sørensen et al. 2006, 2007;



Pastell et al. 2009). Due to its heterogenous structure, the enzymatic degradation of AX requires coordinated action of several different enzymes: Endo-1,4- $\beta$ -xylanases (xylanases, EC 3.2.1.8) catalyze the hydrolysis of the  $\beta$ -1,4 linkages in the backbone of AX (and other xylans), generating a mixture of xylo-oligosaccharides (Biely 1985).  $\beta$ -Xylosidases (EC 3.2.1.37) catalyze the cleavage of xylobiose and the hydrolytic liberation of the terminal xylose unit from the non-reducing end of the xylo-oligosaccharides arising from endo-1,4- $\beta$ -xylanase activity (Rasmussen et al. 2006). Enzymes exhibiting  $\beta$ -xylosidase activity are categorized into seven different GH families: 3, 30, 39, 43, 51, 52 and 54 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Certain bifunctional  $\beta$ -xylosidases may also exert  $\alpha$ -L-arabinofuranosidase activity (and vice versa)—those are mainly categorized in GH family 3 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009).

$\alpha$ -L-Arabinofuranosidases (EC 3.2.1.55), categorized in GH families 3, 10, 43, 51, 54, and 62, catalyze the hydrolysis of terminal non-reducing  $\alpha$ -L-arabino-furanosyl residues from the xylopyranosyl residues in AX and may also catalyze the hydrolytic release of terminal  $\alpha$ -L-arabinofuranosyl residues from other structures (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). A particular issue in relation to arabino-xylans is the presence of similarly linked  $\alpha$ -L-arabinofuranosyls that require different  $\alpha$ -L-arabino-furanosidase activities depending on whether the  $\alpha$ -L-arabinofuranosyls are linked to singly or doubly substituted xylose residues or are linked via ( $\alpha$ 1  $\rightarrow$  2) or ( $\alpha$ 1  $\rightarrow$  3)-links to xylose (Sørensen et al. 2007).

We here report on the finding of endogenous  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities in flax seed mucilage. To our knowledge, this is the first time that the presence these endogenous activities in FM have been reported.

## Materials and methods

### Chemicals

Xylo-oligosaccharide standards (X2, X3, X4, X5 and X6) from birchwood xylan were purchased from Megazyme (Bray, Ireland). Pullulan with average molecular weights of  $0.13 \times 10^4$ ,  $0.6 \times 10^4$ ,  $1 \times 10^4$

and  $40 \times 10^4$  g mol<sup>-1</sup> were from Fluka Chemie AG (Buchs, Switzerland).

### Substrates

Water-unextractable arabinoxylan (WUAX) (arabinose:xylose (A/X): 0.61) and water-extractable arabinoxylan (WEAX) medium viscosity (A/X: 0.61), both from wheat, were obtained from Megazyme (Bray, Ireland). Birchwood xylan was purchased from Sigma-Aldrich and used as is. *p*-nitrophenyl  $\beta$ -D-xylopyranoside (*p*NPX) and *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside (*p*NPA) were obtained from Sigma-Aldrich (Bornem, Belgium).

### Extraction of flax seed mucilage

Brown species of flax seed were bought from Sun Island (JAN Import A/S, Hadsted, Denmark). The mucilage was extracted from the flax seeds with water for 24 h at ambient temperature according to Naran et al. (2008).

### Enzymes

Viscozyme L9 was obtained from Novozymes (Bagsværd, Denmark). This enzyme preparation contained different enzymes including arabanase, cellulase,  $\beta$ -glucanase, hemicellulase, and xylanase (Sørensen et al. 2003). The *Bacillus subtilis* xylanase (BsX) was obtained from Danisco A/S (Brabrand, Denmark). BsX carries a Swiss Prot entry P18429, XynA\_BACSU and has been classified as a glycosyl hydrolase family 11 xylanase according to sequence homology (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). The xylanase was purified from a Grindamyl H 640 enzyme preparation according to Sørensen and Sibbesen (2006). BsX had a molecular weight of 20 kDa, pH optimum at 6, and temperature optimum at 50°C.

### SDS-PAGE

Protein content was analyzed by SDS-PAGE using a Criterion XT Precast Gel, 12% Bis-Tris (Bio-Rad, Hercules CA, US). SDS-PAGE was run at 200 V for 60 min. The running buffer was XT MOPS (Bio-Rad) and the gel was stained with Bio-Safe Coomassie (Bio-Rad) according to the manufacturer's procedure.



## Enzymatic hydrolysis of FM and other xylan substrates

Enzyme-catalyzed hydrolysis, by BsX, of the four substrates (FM, WEAX, WUAX, and birchwood xylan) was done as described in Rasmussen et al. (2010): Substrate (1% dry wt), 0.1 M sodium acetate pH 6 with 0.02% sodium azide. BsX was added to the final reaction at  $0.12 \mu\text{mole} \times \text{enzyme g}^{-1}$  DM substrate. Samples were withdrawn at set times during the incubation and the enzymatic hydrolysis was stopped immediately by heating ( $100^\circ\text{C}$  for 10 min). After centrifugation, the supernatant was analyzed further. For studies of the endogenous activities in FM, the FM was preincubated for 10 min at  $40^\circ\text{C}$  with the substrates (WEAX, WUAX, and birchwood xylan) prior to addition of BsX. The final concentration of FM was  $0.5 \text{ mg DM ml}^{-1}$ . All procedures were performed in triplicate against a reference (no enzyme).

## Enzyme assay and kinetics

Enzyme activity in the FM was assayed by the hydrolysis of 44 mM *p*NPX and *p*NPA, respectively in 0.1 M sodium acetate, pH 6 at  $40^\circ\text{C}$  for 30 min. FM was added in amounts sufficient to promote an increase in absorbance between 0.1 and 1 at 410 nm. The reaction was terminated by the addition of 1 M  $\text{Na}_2\text{CO}_3$ . One unit (U) of  $\beta$ -xylosidase or  $\alpha$ -L-arabinofuranosidase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1  $\mu\text{mol}$  of *p*NPX or *p*NPA  $\text{min}^{-1}$ , respectively. Specific activities are given as units (U) per mg total protein in the FM. For determination of the  $K_M$  values for the  $\beta$ -xylosidase and arabinofuranosidase activities in FM, activity assays were performed on *p*NPX and *p*NPA at concentrations between 3.7 and 44 mM at pH 6 and  $40^\circ\text{C}$ .  $K_M$  and  $V_{\text{max}}$  values for both substrates were calculated from Hanes plots, i.e.  $[\text{S}]/v_i$  vs.  $[\text{S}]$ .

## High performance size exclusion chromatography (HPSEC)

HPSEC was carried out as described in Rasmussen and Meyer (2010).

## High performance anion exchange chromatography (HPAEC)

Separation and quantification of xylo-oligosaccharides in the hydrolysates were performed as described in Rasmussen et al. (2010).

## Results

### Composition of flax seed mucilage (FM)

Determination of the monosaccharide content of FM by HPAEC (Table 1) revealed that xylose was the most abundant monosaccharide of the FM constituting 43% (equivalent to 47 mol %) of the carbohydrate fraction. Galactose was the second most abundant monosaccharide making up about 20% by weight (17 mol %) of the carbohydrate fraction, with arabinose and galacturonic acid at relatively lower levels, and fucose, rhamnose and glucose present in lower amounts (Table 1). By summing up xylose, galactose, arabinose, fucose, and glucose the neutral polysaccharides of flax seed mucilage (FM) were estimated to make up about 83% by weight (equivalent to 85 mol %) of the carbohydrates. Acidic polysaccharides calculated as rhamnogalacturonan 1 were estimated to constitute about 17% by weight with rhamnose and galacturonic acid as the main constituents. The proportion of acidic to neutral polysaccharides was similar to that previously reported by Warrand et al. (2003) and Guilloux et al. (2009), but the A/X ratio of 0.31 was slightly lower than that reported by Guilloux et al. (2009) (they reported A/X 0.36).

The FM also contained about 9.5% by (w/w) protein as determined by a BCA Protein Assay Kit (Table 1). The protein was also seen when a sample of FM was analyzed by SDS-PAGE, where a smear of protein bands occurred, but with a band between 20 and 25 kDa being predominant (data not shown).

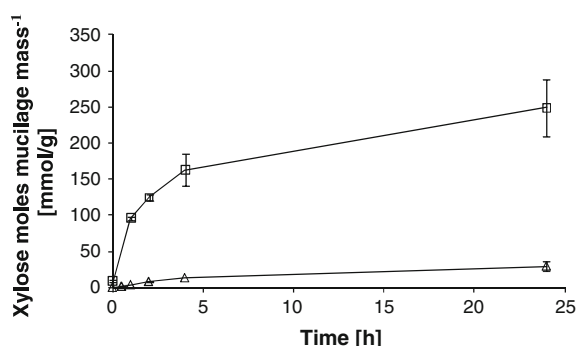
### Enzymatic hydrolysis of FM

During treatment with BsX the reducing ends assay showed a modest increase in xylose equivalents released from FM as compared to from WEAX as a result of the xylanase activity (Fig. 1). The low degree of hydrolysis of FM was also observed with

**Table 1** Monosaccharide content of FM as determined by HPAEC-PAD analysis (Arnous and Meyer 2008) after acid and enzymatic hydrolysis (Garna et al. 2004)

	mg g <sup>-1</sup> DM	mmol g <sup>-1</sup> DM	Distribution (wt%) within the carbohydrate fraction	Distribution (mol %) within the carbohydrate fraction
Xylose	281 ± 9	2.13 ± 0.07	43.1 ± 0.8	46.8 ± 0.7
Galactose	127 ± 3	0.78 ± 0.13	19.5 ± 0.3	17.2 ± 2.1
Arabinose	87 ± 0.9	0.66 ± 0.01	13.3 ± 0.5	14.5 ± 0.5
Fucose	32 ± 0.5	0.22 ± 0.00	4.9 ± 0.2	4.8 ± 0.2
Rhamnose	33 ± 1.3	0.23 ± 0.01	5.1 ± 0.1	5.0 ± 0.1
Galacturonic acid	78 ± 2.9	0.45 ± 0.02	12.1 ± 0.8	9.8 ± 0.6
Glucose	14 ± 0.6	0.09 ± 0.00	2.1 ± 0.1	1.9 ± 0.1
Protein	95			
A/X ratio	0.308			

The data are expressed as mg dehydrated monosaccharide g<sup>-1</sup> dry matter (DM), mmole dehydrated monosaccharide g<sup>-1</sup> DM, and as relative percentage weight and molar distributions, respectively, of the carbohydrate fraction. Arabinose/xylose ratio (A/X) as calculated from HPAEC data and protein content as determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, US)



**Fig. 1** Progress curves of formation of reducing ends assessed as xylose equivalents (xylose moles/mucilage mass [mmol/g]) during the enzyme catalyzed hydrolysis by BsX of FM (*open triangle*) and WEAX (*open square*). The reducing ends were measured by the 4-hydroxybenzoic acid hydrazide (PAHBAH) assay with absorbance measurements at 410 nm (Lever 1977). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation

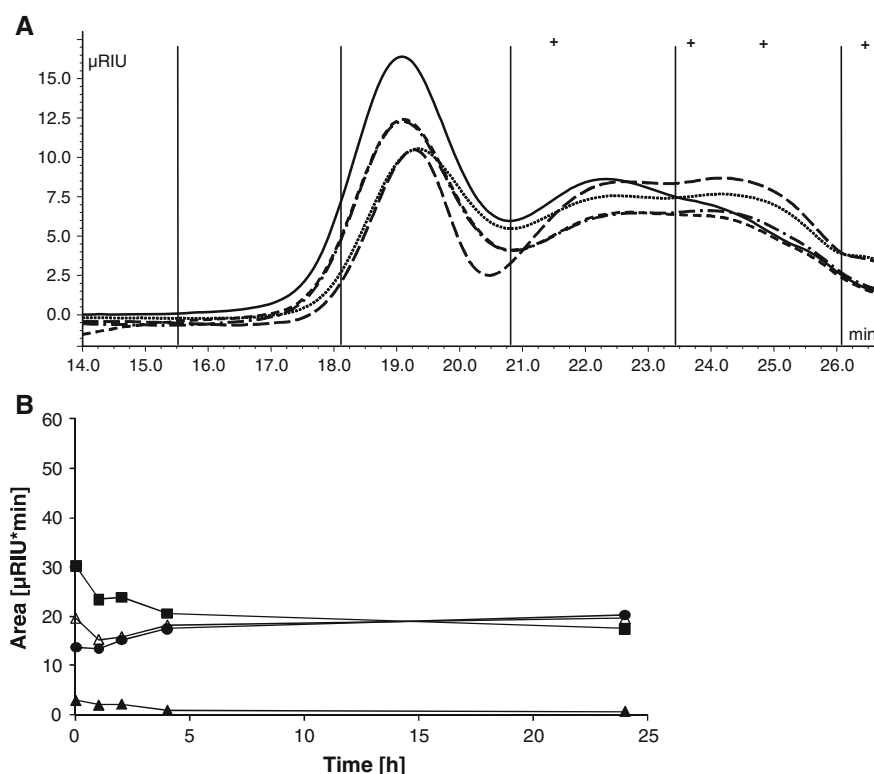
HPSEC where only a slight change in the molecular weight took place during enzymatic hydrolysis as compared to the hydrolysis of WEAX (Figs. 2a, 3a). In order to analytically quantify the progress of the enzymatic hydrolysis, the HPSEC chromatograms were divided into fixed time intervals and the refractive index response (the area under the curve) was used for quantification (Rasmussen and Meyer 2010) (Figs. 2b, 3b). The main change in area on both substrates, representing the effect of the BsX activity, took place during the first 4 h. Thereafter, no

significant change was observed (Figs. 2b, 3b), but the evolution of the area response levels within the time intervals differed for the two substrates (Figs. 2b, 3b). Analysis by HPAEC of the activity of BsX on FM also revealed that the BsX was unable to catalyze the release of significant amounts of small xylooligosaccharides (DP 2-6) from FM during 24 h of incubation (data not shown).

#### Endogenous activity

In order to examine if FM contained any endogenous activities or inhibitors for the BsX, FM was added together with BsX to different AX substrates which differed in structure: WEAX, WUAX and birchwood xylan. In all three cases significant amounts of small XOS (DP 2-6) were released from the substrates during 24 h of incubation (Fig. 4). The XOS were mainly contributed by xylobiose (X2), xylotriose (X3) and to some extent xylotetraose (X4), but xylose was released from all substrates and arabinose from WEAX and WUAX (Fig. 4). Previous results have shown that this BsX xylanase catalyzes the liberation of significant amounts of small XOS (DP 2-6) from these substrates, but no xylose or arabinose (Rasmussen et al. 2010). Therefore, the observed release of xylose and arabinose suggested the presence of endogenous  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities in the FM. Further analysis revealed that initially the arabinose was released

**Fig. 2** BsX catalyzed hydrolysis of FM. **a** HPSEC chromatographic profile after 0 min (—), 1 h (---), 2 h (- · -), 4 h (·····) and 24 h (— — —). Molecular weight markers (+) from left to right are:  $400 \times 10^3$ ,  $110 \times 10^3$ ,  $10 \times 10^3$ , and  $1.3 \times 10^3 \text{ g mol}^{-1}$ . Vertical lines in the chromatogram represent the time intervals used in (b). **b** Progression curves of the area in the following time intervals: 15.5–18.1 min (black triangle), 18.1–20.8 min (square), 20.8–23.4 min (white triangle), 23.4–26.1 min (circle) (no peaks were seen beyond 26 min)

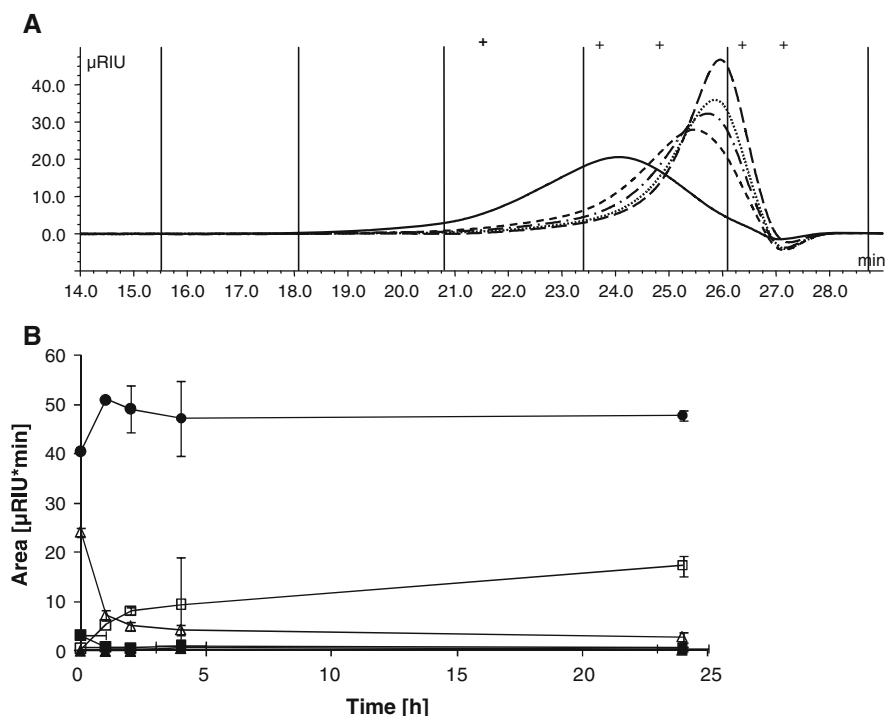


faster than the xylose from both substrates. Nonetheless, after 24 h more xylose than arabinose had been released (Fig. 4). This pattern was confirmed when the initial rates of arabinose, xylose and XOS release in the reactions with BsX and FM added together were compared for all three substrates (Table 2).

The finding that more xylo-oligosaccharides, xylose, and arabinose were released from WUAX than from WEAX during the combined BsX and FM catalyzed hydrolysis (Fig. 4b, c) is in accordance with previous data signifying a higher propensity of BsX to attack WUAX than WEAX (Rasmussen et al. 2010). The highest amounts of XOS and xylose were released from birchwood xylan (Fig. 4a). This can be related to the structure of this substrate since it has a much lower substitution degree than WEAX and WUAX hence presumably providing better access for the BsX enzyme to the xylan backbone. Moreover, the xylose content in birchwood xylan was higher than 90% (by weight) where WEAX and WUAX, both had an A/X ratio of 0.61. No arabinose was released from birchwood xylan which is accordance with the low (negligible) arabinose levels in

birchwood xylan and confirming that while the BsX action on this substrate produced attack points for the  $\beta$ -xylosidase activity in the FM there were hardly any bonds to attack for the putative  $\alpha$ -L-arabinofuranosidase activity.

The effect of the addition of FM to the BsX endo-1,4- $\beta$ -xylanase reaction on WEAX was also evident on the HPSEC chromatogram when compared to that without addition of FM (Figs. 3a, 5a). According to Fig. 2a, the FM contained polymers with a molecular weight larger than  $400 \times 10^3 \text{ Da}$  and these polymers were also seen in Fig. 5a (appear less pronounced because of the scale difference on the vertical axes). During the incubation period, the molecular weight of WEAX changed as result of the endo-1,4- $\beta$ -xylanase treatment, and an increase in low molecular weight molecules was also observed. These smaller molecules were not released when the WEAX was treated with the BsX alone. The areas in the 23–26 min and 26–29 min intervals changed most significantly during the incubation (Figs. 3b, 5b). The decrease in the 23–26 min interval after 4 h incubation indicated that the molecules eluting within this time interval were



**Fig. 3** BsX catalyzed hydrolysis of WEAX. **a** HPSEC chromatographic profile after 0 min (—), 1 h (---), 2 h (- · -), 4 h (·····) and 24 h (— — —). Molecular weight markers (+) from left to right are:  $400 \times 10^3$ ,  $110 \times 10^3$ ,  $10 \times 10^3$ ,  $1.3 \times 10^3$  g mol<sup>-1</sup>, and xylose (150 g mol<sup>-1</sup>). Vertical lines in the chromatogram represent the time intervals

used in **(b)**. **b** Progression curves of the area in the following time intervals: 15.5–18.1 min (black triangle), 18.1–20.8 min (black square), 20.8–23.4 min (white triangle), 23.4–26.1 min (circle), 26.1–28.7 (white square). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation

substrates for the enzyme activities in FM since this decrease was not observed when WEAX was hydrolyzed by BsX alone. Furthermore, the increase in the area between 26 and 29 min, representing molecules smaller than  $1.3 \times 10^3$  g mol<sup>-1</sup>, was 46% higher than the area in the same time interval when hydrolysis of WEAX was catalyzed by BsX without addition of FM.

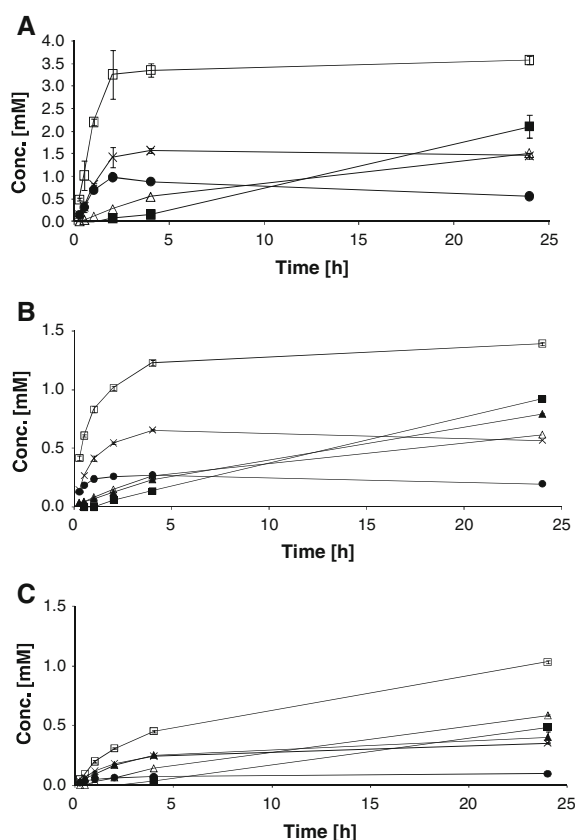
#### Kinetic parameters

The putative endogenous  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities in FM were assayed on *p*NPX and *p*NPA substrates in order to estimate the specific activity and kinetic parameters (Table 3). The specific activities were calculated based on the total protein concentration in FM. Both the specific activity and the substrate affinity (as judged from the

$K_M$  values) were higher on *p*NPX than on *p*NPA (Table 3). These findings contrast the HPAEC results that indicated that the putative  $\alpha$ -L-arabinofuranosidase activity in FM catalyzed a faster release of arabinose than xylose on the genuine polymeric substrates (Fig. 4). The discrepancy may be due to the generally faster rate of  $\beta$ -xylosidase on short oligomers, xylobiose and *p*NPX than on longer AX chains. Nonetheless, since the  $\beta$ -xylosidase activity had a higher specific activity and also higher maximal velocity, more xylose than arabinose was released after 24 h incubation (Fig. 4).

#### Discussion

Previous analysis of the BsX catalyzed hydrolysis of WEAX, WUAX, wheat bran, and birchwood xylan have confirmed that this enzyme is a pure endo-1,



**Fig. 4** Progress curves of the products generated from the catalyzed hydrolysis of different xylan substrates by BsX ( $0.12 \mu\text{mol g}^{-1}$  DM substrate) in the presence of FM ( $0.5 \text{ mg DM} \times \text{ml}^{-1}$ ). **a** WEAX. **b** WUAX. **c** Birchwood xylan. Reaction: 1% weight/volume substrate in sodium acetate buffer of pH 6 at  $40^\circ\text{C}$ . (black triangle): arabinose, (black square): xylose, (black circle): xylobiose, (times): xylotriose, (circle): xylotetraose, (white square): xylo-oligosaccharides (DP 2–6). Values represent the mean of three independent experiments and are shown  $\pm 1$  standard deviation

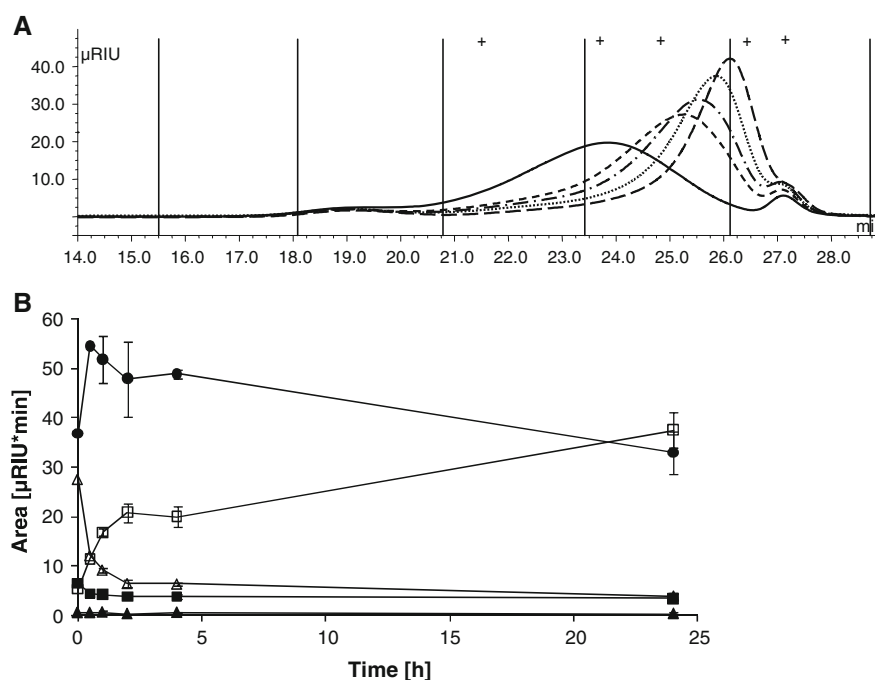
**Table 2** Initial rates ( $v_i$ ) ( $\mu\text{M min}^{-1}$ ) of arabinose, xylose and XOS release by the hydrolysis of individual polymeric substrates by co-incubation with BsX and FM

	$v_i$		
	Arabinose	Xylose	XOS
WEAX	$1.8 \pm 0.13$	$0.3 \pm 0.01$	$3.1 \pm 0.02$
WUAX	$1.5 \pm 0.02$	$0.5 \pm 0.01$	$20.3 \pm 0.28$
Birchwood xylan	<sup>a</sup>	$1.1 \pm 0.03$	$34.1 \pm 0.72$

<sup>a</sup> No arabinose was released from this substrate

4- $\beta$ -xylanase that catalyzes the generation of significant amounts of small XOS (DP 2–6) without any liberation of xylose (or arabinose) from these

substrates (Rasmussen et al. 2010). The finding that BsX treatment on FM only produced a limited degradation—as judged from the evolution of reducing ends—was presumably a consequence of the unique substitutions of the AX in the FM. The wheat AX employed for comparison had an A/X ratio of 0.61 with a distribution of doubly and singly substituted xylopyranosyls of approximately 50:50 and no other main chain substitutions (Sørensen et al. 2007). The compositional analysis of FM showed an A/X ratio of only  $\sim 0.31$ . However, relatively high levels of galactose to xylose were found (Table 1), and previous data have shown that the flax seed FM contains D-galactose (Naran et al. 2008). Guilloux et al. (2009) also suggested that the high ramification of flax seed AX polysaccharides could decrease the efficiency of enzyme catalyzed hydrolysis of flax seed AX. Co-incubation of FM and BsX produced xylose from birchwood xylan and both xylose and arabinose from the wheat arabinoxylan substrates (Fig. 4). This indicated that FM contained  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities that catalyzed the release of xylose and arabinose, respectively, from the xylan substrates. The presence in FM of these endogenous enzyme activities were confirmed by the activity measurements on pNPX and pNPA, respectively. The presence of active enzymes in the FM is most likely related to the extraction method where in this study, the mucilage was extracted at room temperature. In other studies of FM extraction was done at significantly higher temperatures, i.e.  $80^\circ\text{C}$  (Guilloux et al. 2009) and  $100^\circ\text{C}$  (Diederichsen et al. 2006). The concentrations (and/or activities) of these endogenous enzymes were apparently relatively low and only a smear of bands and only a relatively low molecular weight band (25–30 kDa) appeared on the SDS PAGE. The approximate molecular weights of the fungally derived enzymes are generally much higher, e.g. approximately 100 kDa for  $\beta$ -xylosidase from *T. reesei* and 60–92 kDa for different fungal  $\alpha$ -L-arabinofuranosidases (Sørensen et al. 2007). Also, relatively high concentrations of pNPX and pNPA and a long incubation period (30 min) were necessary to measure the kinetic parameters. Whether FM contained two different enzymes or rather a bi-functional  $\beta$ -xylosidase/ $\alpha$ -L-arabinofuranosidase requires further study. Previous results have suggested the existence of bi-functional  $\beta$ -xylosidase/ $\alpha$ -L-arabinofuranosidase enzymes, e.g. in roots of alfalfa (Xiong et al. 2007).



**Fig. 5** Enzyme catalyzed hydrolysis of WEAX by BsX and FM together. **a** HPSEC chromatographic profile after 0 min (—), 1 h (---), 2 h (— · —), 4 h (·····) and 24 h (— · — · —). Molecular weight markers (+) from left to right are  $400 \times 10^3$ ,  $110 \times 10^3$ ,  $10 \times 10^3$ ,  $1.3 \times 10^3$ , and xylose. The vertical lines in the chromatogram represent the time intervals

used in (b). **b** Progression curve of the area in the following time intervals: 15.5–18.1 min (black triangle), 18.1–20.8 min (black square), 20.8–23.4 min (white triangle), 23.4–26.1 min (circle), 26.1–28.7 (white square). Values represent the mean of three independent measurements and are shown  $\pm 1$  standard deviation

**Table 3** Kinetic parameters for FM on *p*NPX and *p*NPA substrates  $\pm$  standard deviation ( $n = 3$ )

	Specific activity (U mg <sup>-1</sup> )	K <sub>m</sub> (mM)	V <sub>max</sub> (U min <sup>-1</sup> )
<i>p</i> NPA	0.053 $\pm$ 0.014	20 $\pm$ 2.3	1.1 $\times 10^{-3} \pm 0.1 \times 10^{-3}$
<i>p</i> NPX	0.075 $\pm$ 0.005	2 $\pm$ 1.2	0.9 $\times 10^{-3} \pm 0.2 \times 10^{-3}$

## Conclusion

FM mucilage contains endogenous  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities being active on synthetic substrates like *p*NPX and *p*NPA and natural substrates like birchwood xylan, WEAX and WUAX. Limited degradation of FM by a pure endo-1,4- $\beta$ -xylanase, BsX, was observed, which may be ascribable to the high proportion of substitution of the FM xylan, notably a possible higher galactose substitution degree compared to e.g. wheat arabinoxylan. The finding of these endogenous activities was probably due to the relatively mild extraction method.

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## **Chapter 6 Assessment of the potential for enzymatic production of glucurono-xylo-oligosaccharides from wheat bran**

This chapter is extended in the form of the report:

Assessment of the potential for enzymatic production of glucurono-xylo-oligosaccharides from wheat bran

Louise E. Rasmussen, Jens F. Sørensen, Anne S. Meyer

### **6.1 Key points**

Several studies have suggested a potential prebiotic effect of arabino-xylo-oligosaccharides (AXOS) and notable various AX hydrolysates. However, there is still a lack of information on the influence of the detailed structure of the putative prebiotic AXOS on the growth of probiotic bifidobacteria (Grootaert et al., 2007, Vardakou et al., 2008). It would be useful, if the detailed structural features of AXOS could be related to their behavior in fermentation studies. This would lead to a better understanding and control of the mechanisms involved. The purpose of the following report was to examine the potential of isolating GXOS from wheat bran. This work was based on the hypothesis that the presence of glucopyranosyl uronic acid substitution might boost the bifidobacterial response. A first prerequisite was to purify GXOS from a source, potentially rich glucurono-arabinoxylan e.g. wheat bran.

The isolation was done by the following experimental set up as depicted in Figure 11. The GXOS were purified from wheat bran via specific treatment with BsX<sub>mut</sub>. After the enzyme catalyzed hydrolysis, the GXOS were isolated by anion exchange chromatography and the fractions obtained were analyzed for the presence of uronic acid, and by HPAEC and LC/MS for structural verification. Since phosphate co-eluted in the anion exchange chromatography, the amount of phosphate in the fractions was also determined.



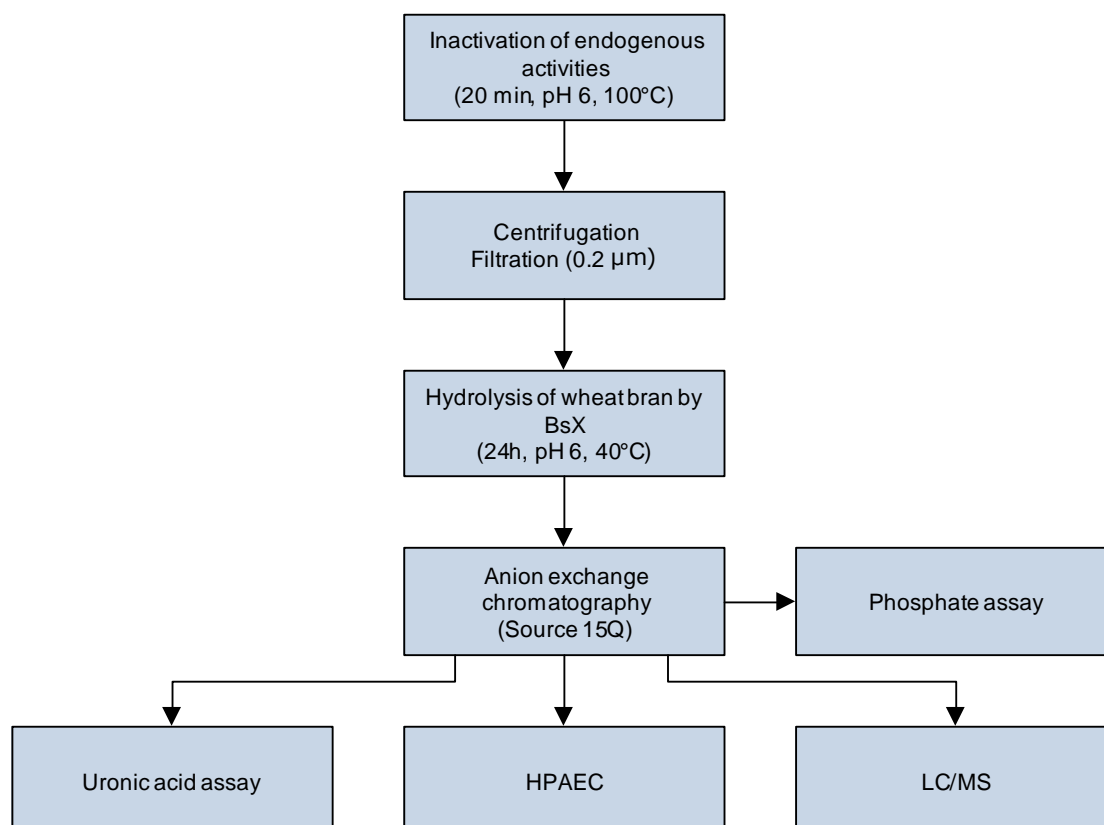


Figure 11. Experimental set up for the purification and characterization of glucurono-xylo-oligosaccharides (GXOS).

### Uronic acid assay

The amount of uronic acid in the fractions obtained after anion separation was quantified colorimetrically by the Blumenkrantz and Asboe-Hansen m-hydroxydiphenyl (MHDP) assay. In this assay, MHDP reacts with uronic acid to produce a pink-brownish compound with an absorbance maximum at 520-530 nm. This assay was used as an indicator for the presence of GXOS. However, it should be noted that a distinction between the different uronic acids (galacturonic, glucuronic, and mannuronic acid) is not possible with this method (van den Hoogen et al., 1998).

In order to determine the uronic acid content by colorimetry, the uronic acid units must be freed from the polymer by acid hydrolysis. Applying a strong acid (most often sulphuric acid) and high temperature is a necessary initial step for the hydrolysis of the glycosidic bonds. However, a drawback of this procedure with biological samples is, that neutral sugars are converted into brown products compromising the specificity of the assay (van den Hoogen et al., 1998). In order to minimize the interference by neutral sugars, sulfamate is added to the sample before the first heating in sulphuric acid, which greatly lowers the browning (Filisetti-Cozzi and Carpita, 1991). Sodium tetraborate is also added to increase the sensitivity of detection of certain uronic acids (Filisetti-Cozzi and Carpita, 1991).

The presence of protein in the samples also interfere with the specificity of the assay and protein levels in the sample higher than 0.25% (w/v) are detrimental for the assay (van den Hoogen et al., 1998).

Anion exchange chromatography was used for purification of GXOS but the results showed that phosphate, most likely originating from phytic acid, and RGI oligomers were also isolated by this method. Phytic acid and RGI will therefore be described in the following sections.

### Phytic acid

Phytic acid (phytate; *myo*-inositol 1,2,3,4,5,6, hexakisphosphate) consists of a phosphorylated *myo*-inositol ring and is the primary source of inositol and storage phosphorus in plant seeds (Figure 12). It contributes up to 80% of total phosphorus and as much as 1.5% to the seed dry weight (Bohn et al., 2007). The strong chelating characteristic of phytic acid reduces the bioavailability of other essential dietary nutrients such as minerals (e.g.  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+/3+}$ ), proteins and amino acids (Bohn et al., 2007). Phytate mainly accumulates in protein storage vacuoles as globoids, predominantly located in the aleurone layer (wheat, barley and rice) or in the embryo (maize). During germination, phytate is hydrolyzed by endogenous phytases (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.26) and other phosphatases to release phosphate, inositol and micronutrients to support the emerging seedling (Bohn et al., 2008). The abundance of phytic acid in cereal grains is a concern in the foods and animal feeds industries because the phosphorus in this form is unavailable to monogastric animals due to lack of endogenous phytases.

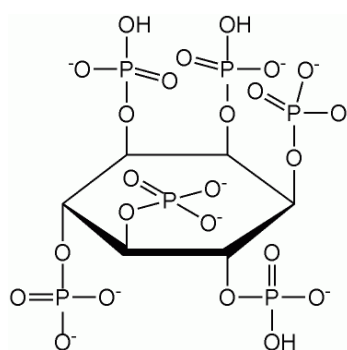
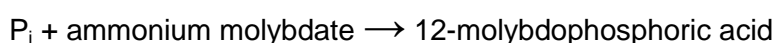


Figure 12. Structural formula of phytic acid.

Phytic acid is quantified by using a colorimetric method, where the phytic acid first is converted to phosphate by treatment with a phytase and subsequently with an alkaline phosphatase. The phosphate ( $\text{P}_i$ ) reacts with ammonium molybdate to form 12-molybdophosphoric acid, which is subsequently reduced under acidic conditions to molybdenum blue:



12-molybdophosphoric acid + H<sub>2</sub>SO<sub>4</sub> / ascorbic acid → molybdenum blue

The amount of molybdenum blue formed in this reaction is proportional to the amount of phosphorus present in the sample and is measured by the increase in absorbance at 655 nm. This assay can therefore be used to quantify the phytic acid and the phosphate present in a sample. The phosphorus is quantified from a calibration curve generated using standards of known phosphorus concentrations (~0.5 to ~7.5 µg).

### **Pectin**

Pectins is a collective name for a mixture of heterogeneous, branched, and highly hydrated polysaccharides present in the plant cell walls. This group of polymers is largely restricted to the primary cell wall, accounting for about one third of all primary cell wall macromolecules. The basic structure consists of “smooth” regions of homogalacturonan, and “hairy or ramified regions” of xylogalacturonan, rhamnogalacturonan I (RGI), rhamnogalacturonan II and polysaccharides comprised mostly of neutral sugars, such as arabinan, galactan, and arabinogalactan (Sakamoto and Sakai, 1995; Bacic et al., 1988).

RGI has a heteropolymeric backbone which contains 1,2-linked α-L-rhamnopyranosyl and 1,4-linked α-D-galactopyranosyl uronic acid. This is in contrast to homogalacturonan, xylogalacturonan, rhamnogalacturonan II which are homopolymeric. The rhamnosyl residues are highly substituted at the C(O)-4 position, ranging from 20 to 80% in most cases (Ros et al., 1996). The side chains may vary in sizes from a single residue to 50 or more. Arabinan, galactan, and arabinogalactan are often attached as side chains to the C(O)-4 position of 1,2-linked α-L-rhamnopyranosyl residues of RGI. RGI has not been a typical feature in wheat bran and there have been no earlier reports on the isolation of this polymer from wheat bran.

## **6.2 Conclusion**

The hypothesis for this report was that wheat bran can be used as a potential source for the enzymatic production of GXOS. The obtained results showed that GXOS could be liberated enzymatically and purified by use of anion exchange chromatography from wheat bran. However, even larger amount of RGI was present in the obtained samples together with phosphate. Therefore, further separation has to be made before GXOS can be isolated.

## 6.3 Assessment of the potential for enzymatic production of glucurono-arabino-xylo-oligosaccharides from wheat bran

### Abstract

Arabino-xylo-oligosaccharides (AXOS) generated from the enzyme catalyzed hydrolysis of arabinoxylan have been reported for their ability to stimulate the growth and development of gastrointestinal microflora. To our knowledge, glucurono-xylo-oligosaccharides (GXOS) have not been isolated from wheat bran and we here report on the potential of enzymatic production of GXOS from wheat bran via specific treatment with a engineered *Bacillus subtilis* XynA xylanase (BsX<sub>mut</sub>). The hypothesis underlying this work was that wheat bran was a potential source for the enzymatic production of GXOS. After the enzyme catalyzed hydrolysis by BsX, the GXOS were isolated by anion exchange chromatography and the fractions obtained were analyzed for the presence of uronic acid, and by High Performance Anion Exchange Chromatography (HPAEC) and LC/MS for structural verification. Since phosphate was co-eluting in the anion exchange chromatography, the amount of phosphate in the fractions was also determined. The results showed that GXOS was isolated from wheat bran but an even larger amount of RGI was present in the obtained samples together with phosphate. Therefore, further purification has to be made before GXOS can be isolated.

### Introduction

In the recent years, attention has been paid to the nutritional properties of the hydrolytic degradation products of arabinoxylan, arabino-xylo-oligosaccharides (AXOS) and the non-substituted xylo-oligosaccharides (XOS), since they have demonstrated prebiotic properties. Prebiotics have been defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon, thereby improving host health (Gibson and Roberfroid, 1995). Ingestion of prebiotics typically shifts the composition of the intestinal bacterial population towards a relative increase in *Bifidobacterium* and/or *Lactobacillus* species. This shift in the intestinal microbiota is associated with improved overall health, reduced gut infections, better absorption of minerals, and suppression of colon cancer initiation (MacFarlane et al., 2006; Wong et al., 2006).

The production of (A)XOS from different plant materials can be categorized into three groups: 1. Extraction using an alkaline solution, followed by neutralization and hydrolysis with an xylanase (Yamada et al., 1993; Beaugrand et al., 2004); 2. Aqueous extraction and autohydrolysis under high pressure/high temperature conditions (Garrote et al., 2002; Kabel et al., 2002; Carvalheiro et al., 2004); 3. Aqueous extraction and hydrolysis in the presence of high doses of an xylanase (Beaugrand et al., 2004; Shiiba et al., 2004; Maes et al., 2004;

Swennen et al., 2006). In contrast to autohydrolysis, the xylanase treatment (having low exo-xylanase and/or  $\beta$ -xylosidase activity) is more desirable because it does not produce undesirable by-products or high amounts of monosaccharides and does not require special equipment. Therefore, there are many reports describing the production of (A)XOS by enzymatic hydrolysis of xylan from oat spelt (Chinshuh et al., 1997), beech wood (Freixo and de Pinho, 2002), birch wood (Aachary and Prapulla, 2008), corncob (Ai et al., 2005; Yoon et al., 2006; Aachary and Prapulla, 2009), wheat straw (Swennen et al., 2005), and hardwood (Nishimura et al., 1998).

Acidic xylo-oligosaccharides i.e. xylo-oligosaccharides substituted with glucopuranosyl uronic acid, have been purified from several glucuronoxylan containing substrates like *Eucalyptus globulus* (Togashi et al., 2009), chips of Eucalyptus wood (Kabel et al., 2002), and birch wood (Christakopoulos et al., 2003). Common for these methods were the usage of substrates containing xylan with high glucuronic acid content. For some of these methods, the prebiotic effect was assessed directly on the hydrolysate or the xylo-oligosaccharides were further purified by HPSEC or anion exchange chromatography. To our knowledge, acidic xylo-oligosaccharides have not been isolated from wheat bran. The objective of this study was therefore to assess the potential of the enzymatic production of GXOS from wheat bran via specific treatment with *Bacillus subtilis* Xyn xylanase.

## **Materials and Methods**

### **Chemicals**

All reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium), unless specified otherwise.

### **Substrate**

The wheat bran was obtained from Valsemøllen (Esbjerg, Denmark), which fractionated the original product into the following parts: Flour (80.7%), course bran (4.7%), fine bran (10.9%), shorts (3.2%), and germ (0.5%). The fraction containing the course bran was used for the work presented in this article. The wheat bran was milled to a particle size less than 0.5 mm.

### **Enzyme**

The D11F/R122D variant of the *B. subtilis* XynA xylanase, BsX<sub>mut</sub>, less prone to inhibition by either TAXI was obtained from Danisco A/S (Brabrand, Denmark). BsX<sub>mut</sub> was purified according to Sørensen and Sibbesen (2006). The xylanase had a molecular weight of 20 kDa, pH optimum at 6, temperature optimum at 50°C, and similar pH and temperature stabilities. It is been classified in glycosyl hydrolase (GH) family 11 according to sequence homology (Cantarel et al., 2009).

### ***Acid hydrolysis***

Galactose, arabinose and xylose content were assessed by 0.4 M HCl hydrolysis for 2h at 100°C according to Sørensen et al., 2003. Glucose and glucuronic acid were determined after 72% H<sub>2</sub>SO<sub>4</sub> hydrolysis at 37°C for 1h followed by 4% H<sub>2</sub>SO<sub>4</sub> hydrolysis at 121°C for 2h according to the standard procedure of the U.S. National Renewable Energy Laboratory (NREL) (Pedersen and Meyer, 2009; Sluiter et al., 2010).

### ***Enzymatic hydrolysis of wheat bran***

Enzyme catalyzed hydrolysis of wheat bran, using BsX<sub>mut</sub>, was assessed according to the following procedure: Substrate (20 wt%) was dissolved in 0.1 M sodium acetate pH 6. A suitably diluted enzyme solution was added to give a final reaction concentration of 0.019 g enzyme g<sup>-1</sup> dry matter (DM) substrate. The enzymatic hydrolysis was stopped by heating the sample at 100°C for 10 min. After centrifugation (5000 g, 10 min), the supernatant was filtered through a cup-filter (0.2 µm) and subsequently applied to the anion exchange system.

### ***Ion exchange chromatography***

Anion separation was performed at room temperature with an ÄKTA purifier 100 work station equipped with a P-900 pump, UV-900 monitor, pH/C monitor, and Frac-950 fraction collector, all controlled by UNICORN software. A HR16/10 column with a 23 mL packed bed of high resolution Source 15Q was used for separation. The elution of proteins was monitored by the UV absorption at 280 nm and unsaturated polysaccharides at 235 nm. Elution was performed at a flow rate of 10 mL min<sup>-1</sup> with water and ammonium formate as eluents. Before injection the column was equilibrated with 20 mM ammonium formate for two column volumes (CV). After injection the column was washed with 20 mM ammonium formate for three CV, followed by elution with a gradient specified below. After elution the column was regenerated with 1000 mM ammonium formate for two CV and with water for three CV. To obtain the oligomers, a sample was injected on the column and eluted using a linear gradient from 20-400 mM ammonium formate for 10 CV. Collection of peaks of interest was performed using a level fractionation parameter. Fractionation was performed using an elution volume (2.5 ml) as the fractionation parameter. Quantification of uronic acid and phosphorus was performed on the collected fractions which also were analyzed by HPAEC and LC/ MS.

### ***Quantification of uronic acids***

The colorimetric determination was done according to the method described in van den Hoogen et al., 1998. In brief, 40 µl sample containing 0.5 to 8 µg uronic acid and 200 µl concentrated sulfuric acid (96% (w/w)) containing 120 mM sodium tetraborate was applied to each well of a 96 well microtiter plate. After mixing of the sample and the reagent by three in and out movements with the same pipet, the plate was placed in an incubator for 1h at 80°C.

After cooling to ambient temperature, the background absorbance of the sample was measured at 540 nm. Then 40 µl of m-hydroxydiphenyl reagent (100 µl of m-hydroxydiphenyl in dimethyl sulfoxide, 100 mg ml<sup>-1</sup>, mixed with 4.9 ml 80% (v/v) sulfuric acid just before use) was added and mixed with the samples as described above. After 15 min the absorbance of the samples was read again at 540 nm. A glucuronic acid standard series (0-8 µg) was included with each microtiter plate.

### ***Quantification of phosphorus***

For quantification of phosphorus part of the “Phytic acid (Phytate) / Total phosphorus” assay from Megazyme was used adjusted to microtiter plates. A color reagent was made by solution A: Ascorbic acid (10% w/v) / Sulphuric acid (1M) and solution B: Ammonium molybdate (5% w/v) in the ratio 1 part B was mixed with 5 part A. 200 µl of the sample or phosphorus standard was mixed with 100 µl of the color reagent and the mixture was incubated at 40°C for 1h. Then, the absorbance at 655 nm was read. A phosphorus calibration curve was made (0-7.5 µg).

### ***High Performance Anion Exchange Chromatography (HPAEC)***

Monosaccharides were measured by HPAEC according to Arnous and Meyer, 2008. Separation of acidic oligosaccharides was performed on a system described in Rasmussen et al. (2010). Separation of the acidic oligosaccharides was achieved by using a three-eluent system comprising (A) deionised water (18.2 mΩcm at 25°C), (B) 0.5 M NaOH and (C) 0.5 M NaOAc. Acidic oligosaccharides were separated using the following linear gradient program: 0 min, 80:20:0 (% A:B:C); 5 min, 0:20:80; 20 min, 0:20:80. Glucuronic acid was therefore used as a bench mark under the assumption that oligosaccharides eluting after glucuronic acid was acidic oligosaccharides with a DP higher than 1.

### ***Liquid chromatography-mass spectrometry (LC/MS)***

Prior to LC/MS analysis, samples were desalted on a graphitized porous carbon (GPC) cartridge. The cartridge was first activated with 3x0.5 ml of acetonitrile/0.1% trifluoroacetic acid (80/20) followed by 3x0.5 ml water (Milli-Q). The sample (1 ml) was applied to the column and the salts were removed with 3x0.5 ml water. Neutral oligosaccharides were eluted with 2x0.5 ml acetonitrile/water (50/50). Acidic oligosaccharides were eluted with 2x0.5 ml acetonitrile/0.1% trifluoroacetic acid (80/20). The collected fractions were dried on a SpeedVac. The dried samples were re-solved in 50 µl water and diluted with 50 µl acetonitrile before analysis. The oligosaccharides are by this procedure concentrated 10 times before analysis.

## Results

### *Composition of wheat bran*

Determination of the monosaccharide content of wheat bran by HPAEC revealed that xylose was the most abundant monosaccharide of the wheat bran constituting 44 wt% of the carbohydrate fraction (Table 1). Arabinose and glucose were the second and third most abundant monosaccharide making up about 28% and 25%, respectively, with galactose and glucuronic acid at relatively lower amounts.

Table 1. Monosaccharide content of wheat bran determined by HPAEC analysis after acid hydrolysis. The data are expressed as mg dehydrated monosaccharide g<sup>-1</sup> dry matter (DM), and as relative percentage of the carbohydrate fraction.

	mg g <sup>-1</sup> DM	Distribution (wt%) within the carbohydrate fraction
Xylose	187±2.0	44±1.4
Arabinose	117±2.3	28±0.3
Glucose	104±11.3	25±2.2
Galactose	12±0.3	3±0.0
Glucuronic acid	3±0.9	0.9±0.1
A/X ratio	0.62	

### *Anion separation*

After the catalyzed hydrolysis of wheat bran by BsX<sub>mut</sub>, the hydrolysate was separated by anion exchange chromatography. Arabinoxylan had no absorbance, so only proteins were detected by UV monitoring at 280 nm and unsaturated polysaccharides at 235 nm (Figure 13). Fractions were collected during the separation and the uronic acid content was quantified in each fraction (Figure 13). The uronic acid content was significantly high in the fractions obtained at approximately 15-17 min and 19-23 min in the elution program. In some of these fractions, an increase of UV monitoring at 235 nm (21-23 min) was observed. Also high amounts of uronic acid were measured at the end of the profile (28-30 min).



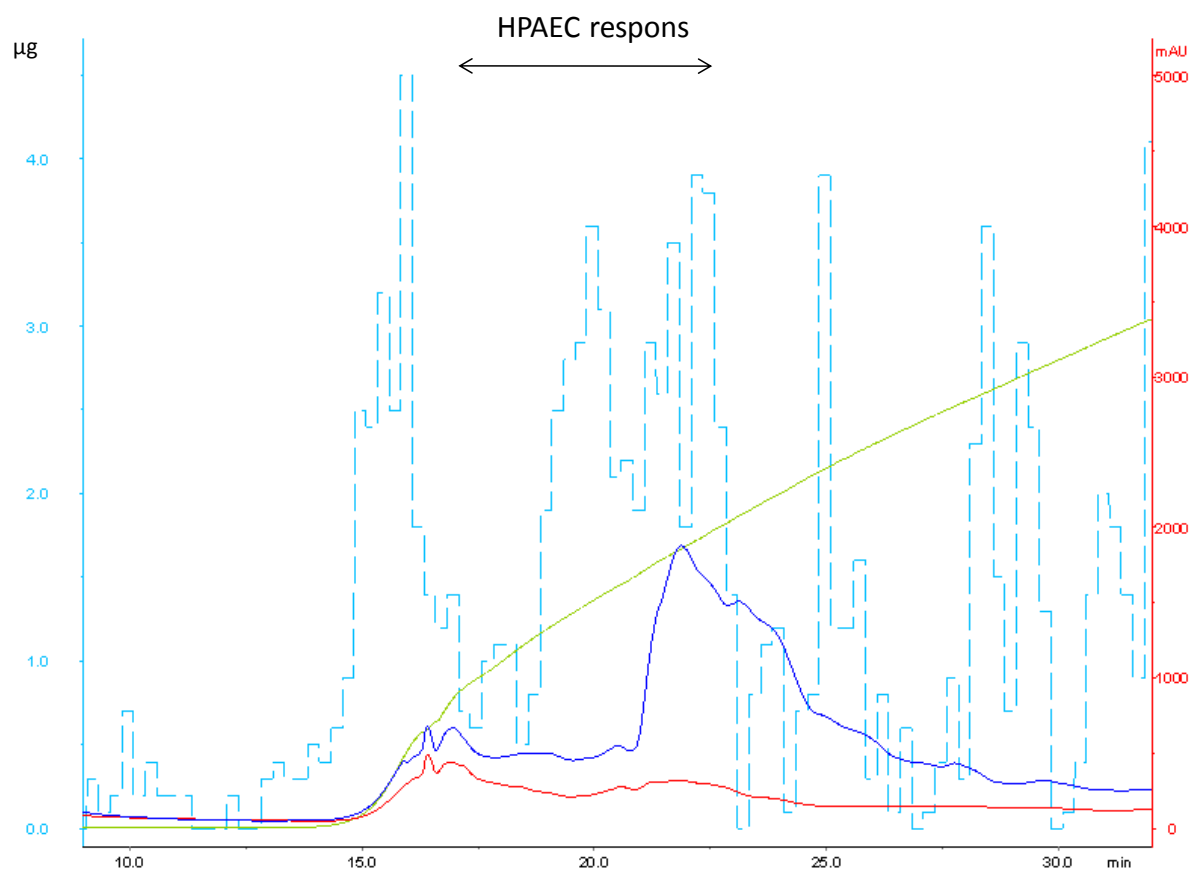


Figure 13. Elution profile during anion exchange chromatography on a Source 15Q column using a linear gradient of ammonium formate and UV detection at 235 and 280 nm. Red: UV detection at 280 nm; Dark blue: UV detection at 235 nm, Light green: Conductivity (0-60 mS cm<sup>-1</sup>); Light blue: Uronic acid [10<sup>-2</sup> µg]. HPAEC response was detection of compounds eluting after glucuronic acid by HPAEC analysis.

Analysis by HPAEC showed that fractions obtained at 17-23 min contained mainly two types of oligomers eluting after glucuronic acid (retention time = 24 min) (Figure 14). These results indicated that the fractions obtained at 17-23 min most likely contain uronic acids oligosaccharides. The HPAEC analysis of the fractions obtained before and after 17-23 min did not detected any response despite the detection of uronic acid by the MHDP assay.

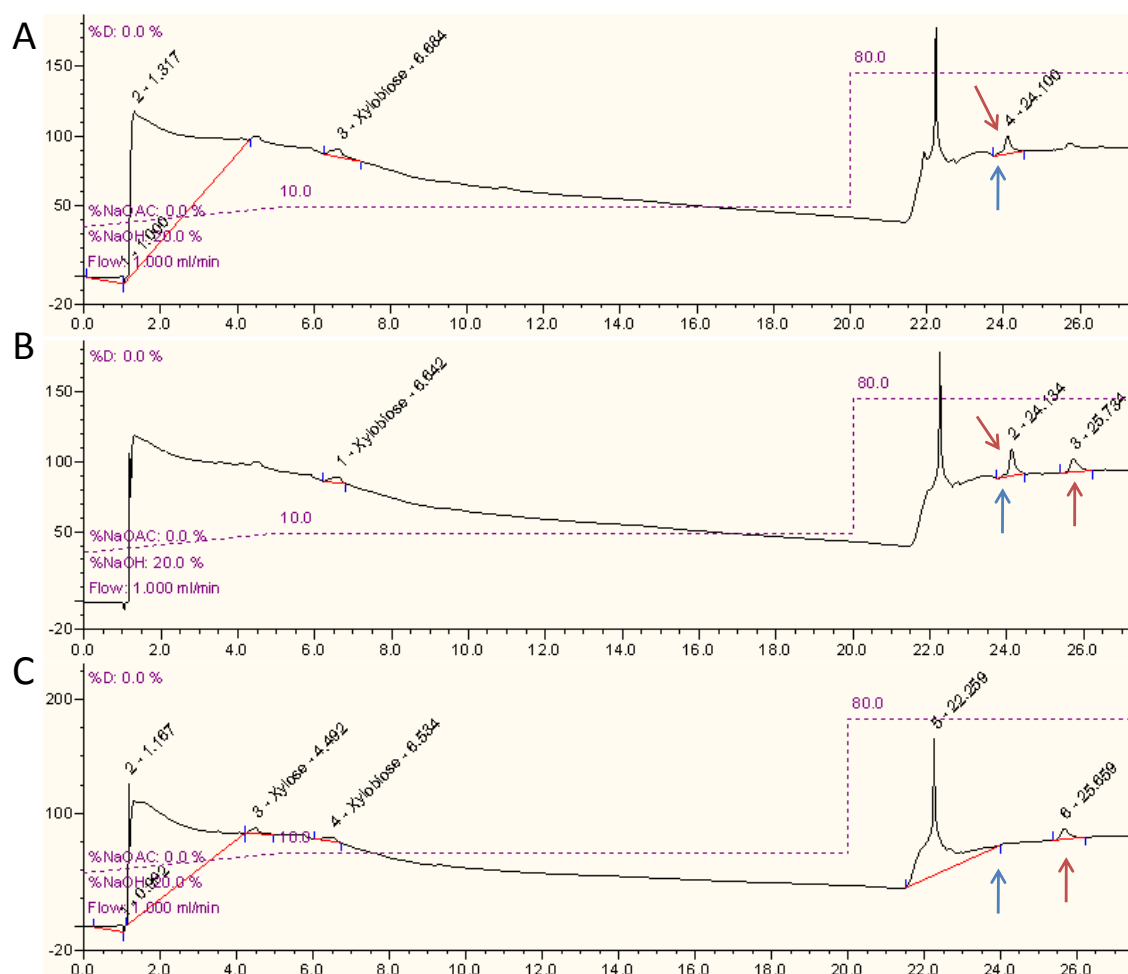
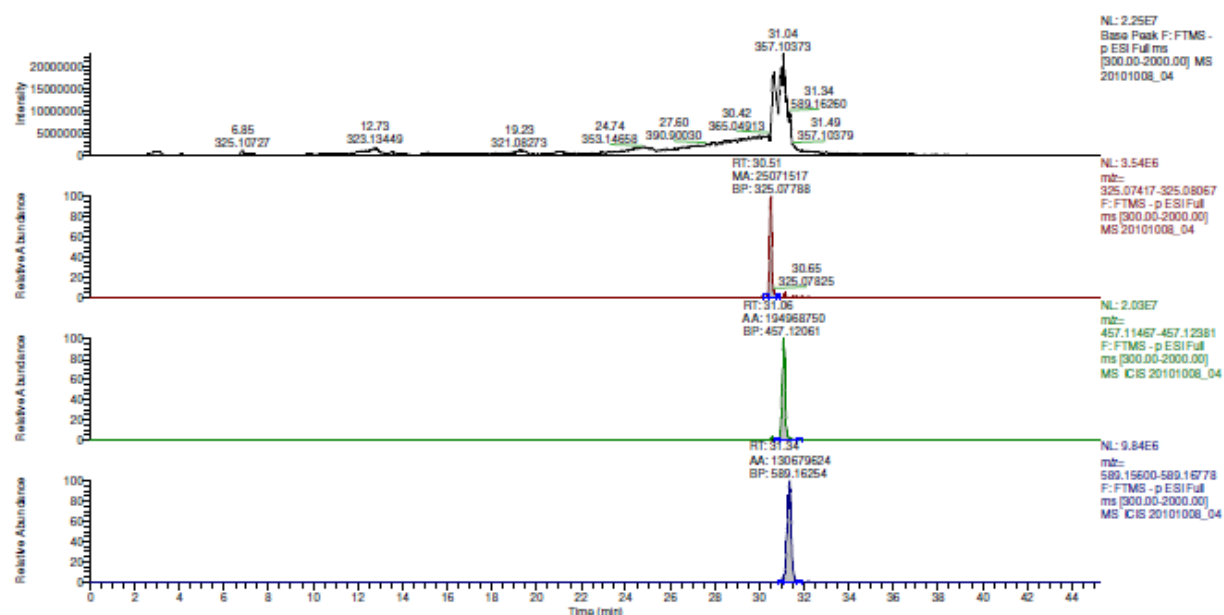


Figure 14. HPAEC chromatograms of 3 fractions obtained after anion separation. A: 18 min, B: 20, C: 22.5 min. Blue arrow: Glucuronic acid – used as bench mark; Red arrow: Uronic oligosaccharides;

The fractions, which gave a HPAEC response, were further analyzed by LC/MS for structural verification (Figure 17). GXOS homologous (DP 2-4) were found in the fraction obtained at 18 min. However, since arabinofuranosyl and xylopyranosyl had the same molecular weight, the LC/MS analysis could not determine, whether the glucopyranosyl uronic acid was linked to an unsubstituted xylo-oligosaccharide or an arabinofuranosyl substituted xylo-oligosaccharide. In the other samples, the GXOS could not be detected. Instead, a significant signal from phosphate or phosphate containing components, probably phytate, was observed. Since phosphate showed a good response in ESI (Electrospray ionization) negative mode, they might cause significant ion suppression of the target components in the mass spectrometer. This could be why, the GXOS could not be detected in the other fractions. The presence of phosphorus in several fractions was confirmed by the molybdenum blue assay (Data not shown).



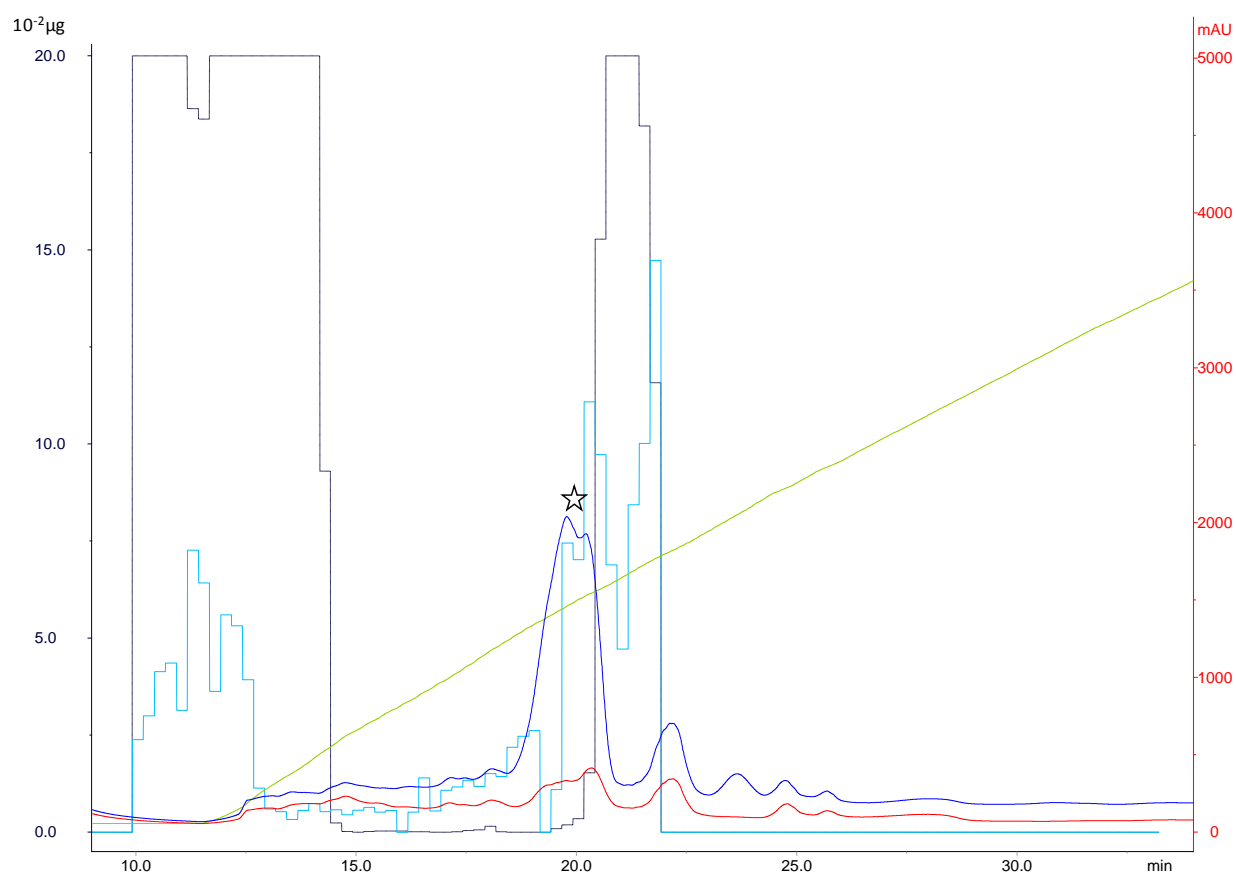


Figure 16. Elution profile during anion exchange chromatography on a Source 15Q column using a linear gradient of ammonium formate and UV detection at 235 and 280 nm. Red: UV detection at 280 nm; Dark blue: UV detection at 235 nm Light green: Conductivity ( $0\text{--}60 \text{ mS cm}^{-1}$ ); Black: Phosphorus [ $\mu\text{g}$ ]; Light blue: Uronic acid [ $10^{-2} \mu\text{g}$ ]. ☆ : Sample analyzed by LC/MS.

When the fraction obtained at 19.5 min was analyzed by LC/MS, the sample was fractionated into neutral and anionic oligosaccharides. From the anionic fraction three major components were detected (Figure 17.A).  $m/z$  366 was a heterocyclic glycoside,  $m/z$  346 a phosphate-compound, while  $m/z$  1287 could be interpreted as a rhamnogalacturonic 4-mér (known from the “hairy” region from hydrolysed pectin) (Figure 17.B).

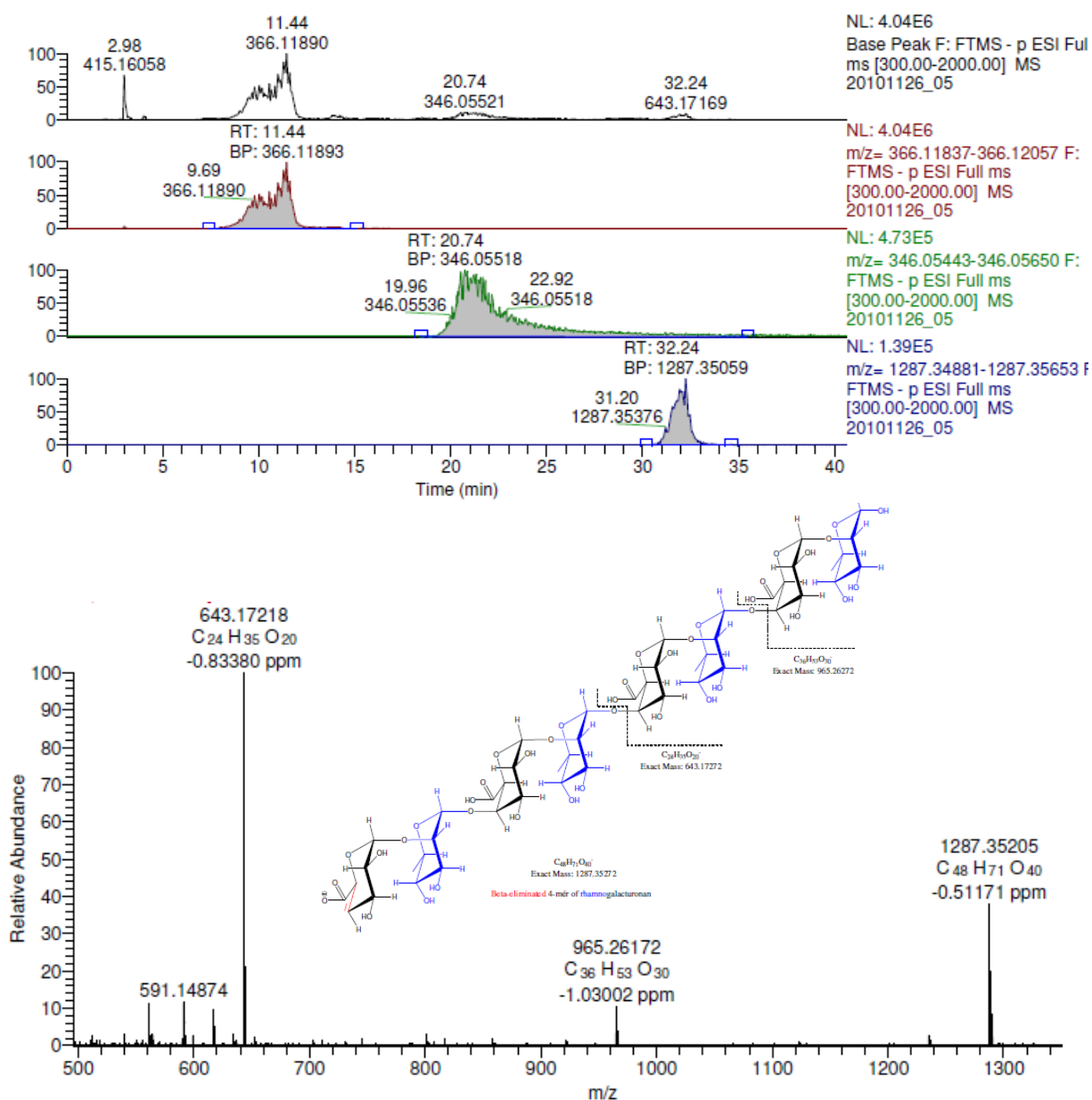


Figure 17. Base-peak chromatogram and extracted ion chromatograms. Red: m/z 366 - heterocyclic glycoside; Green: m/z 346 - phosphate-compound; Blue: m/z 1287 - 4-mér of rhamnogalacturonan (RGI). B. FTMS of peak at 32.08 min. m/z 643: 2-mér of RGI; m/z 965: 3-mér of RGI; m/z 1287: 4-mér of RGI.

Figure 18 shows four components from the neutral fraction, where m/z 366 was the same heterocyclic glycoside found in the anionic fraction. m/z 304 was probably a N-containing compound, while m/z 589 and m/z 721 were GXOS DP 4 and 5. However, it should be noticed that the levels of these two compounds ( $9.07 \times 10^3$  and  $3.23 \times 10^3$ ) were very low compared to m/z 1287 ( $1.39 \times 10^5$ ) and the ratios (m/z 589)/(m/z 1287) and (m/z 721)/(m/z 1287) were 6.5% and 2.3% respectively. This meant that the uronic acid detected by MHDP assay and the HPAEC response primarily came from the m/z 1287 (tentatively explained as RGI).

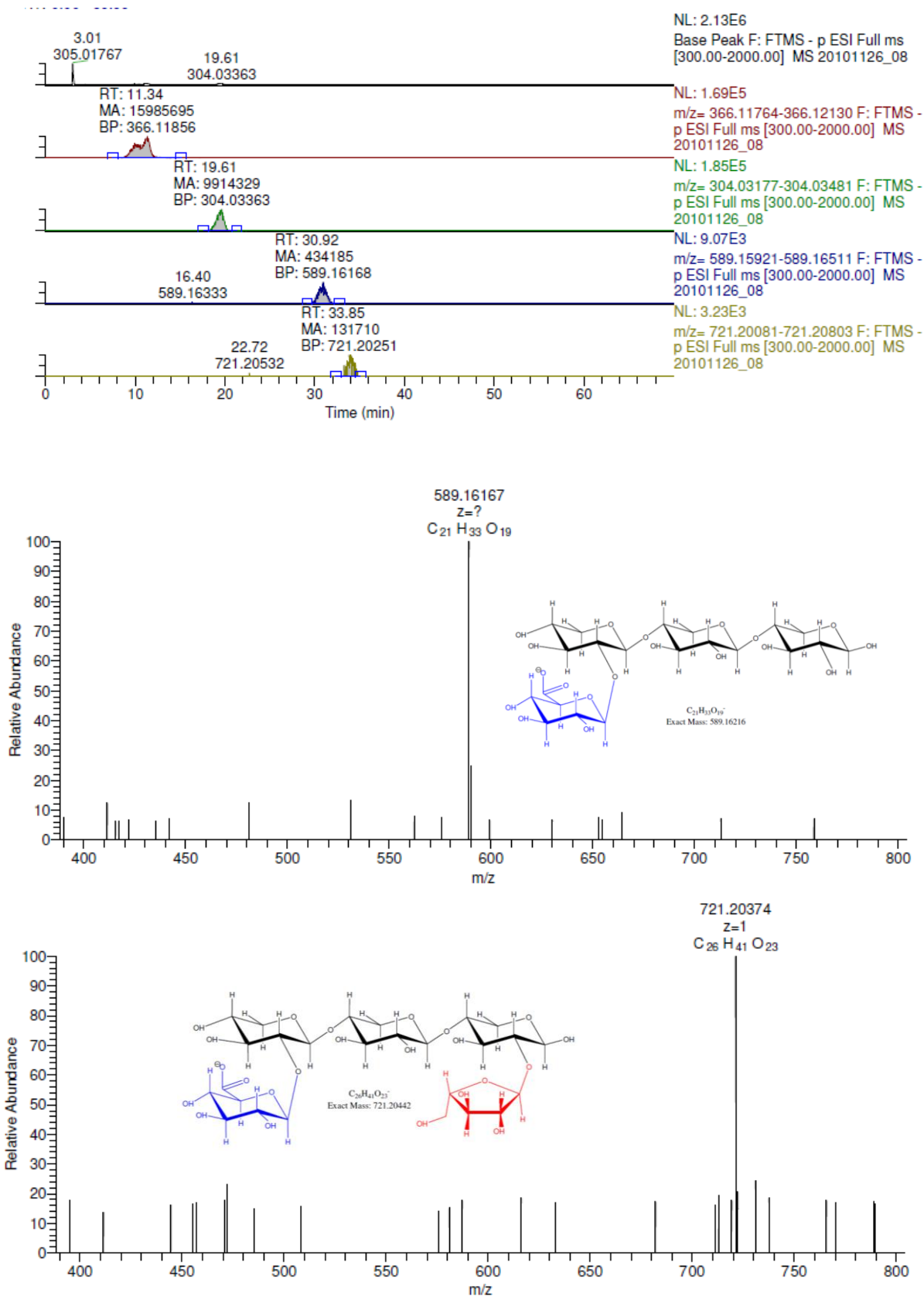


Figure 18 Base-peak chromatogram and extracted ion chromatograms. Red: m/z 366 - heterocyclic glycoside; Green: m/z 304 – N-containing compound; Blue: m/z 589 – GXOS DP4; Yellow: m/z 721 – GXOS DP5. FTMS of peak at 32.08 min. B: m/z 589; C: m/z 721.

So GXOS homologous were detected, but an even larger amount of uronic acid residues, which may come from what is tentatively interpreted as RGI, were present in the sample. Furthermore, the major component of the sample actually seemed to be the heterocyclic glycoside ( $m/z$  366).

## Discussion

In the composition analysis, glucuronic acid was detected but not galacturonic acid (Table 1). This is somewhat surprisingly, since the LC/MS data showed that the concentration of RGI was approximately 22 times higher than that of the GXOS homologous. However, both quantification methods are related with some uncertainties and further analysis is needed for a more precise quantification of both oligosaccharides.

Since the colorimetric determination of uronic acids by the MHDP assay is non-distinctive between the different uronic acids, not only glucuronic acid was quantified in the fractions. Since RGI contained galacturonic acid, this monosaccharide was also quantified with by assay. A method for the distinction between the two uronic acids has been reported by Renard et al., 1999. Apparently, galacturonic acid and glucuronic acid give a different response to the addition of sodium tetraborate. The difference in response of the two uronic acids in the presence and absence of sodium tetraborate, could therefore be used to quantify them within a mixture containing both galacturonic and glucuronic acid. Thus, it would be possible to examine whether the RGI and the GXOS homologous are co-eluting in all the fractions, or whether there has been a separation to a certain degree.

The RGI could be removed from the wheat bran by introducing an initial step, where the wheat bran was first treated with a pectinolytic enzyme preparation, with a subsequent removal of the released mono- and oligosaccharides by e.g. filtration. However, the enzyme treatment should be prior to the addition of the xylanase, since it will be rather difficult to separate the two groups of generated oligosaccharides afterwards.

When GXOS was isolated by anion exchange chromatography, not all fractions could be used due to the presence of phosphate in some of the fractions, which most likely origin from phytic acids in the wheat bran. Despite the inactivation of endogenous phytase activities, there was still phosphate present in the wheat bran. A hypothesis could be, that the wheat bran or kernel had been wetted, with the subsequent activation of the phytase, catalyzing the release of phosphate.

Ammonium formate was used as eluent since this buffer can be removed from the fractions by freeze drying. The pH of ammonium formate was approximately 6.5, which ensured that the glucopyranosyl uronic acid in the GXOS was protonized since the  $pK_a$  value is 3.36. Phosphate has 3  $pK_a$  values: 2, 7, and 12, which meant that at pH 6.5, this compound was

also charged. This explained the co-elution of GXOS and phosphate. Experiments have been executed, in which pretreated wheat bran was washed with either water (4h, 40°C) or ethanol (2h, 80°C) followed by filtration to remove the phosphate (Data not shown). Despite these attempts, the phosphate was still present in similar amounts in the fractions. Thus, other methods have to be considered. However, if the phosphate and the GXOS were to be more separated by anion exchange chromatography, another pH in the buffer should be used in order to obtain a different binding affinity of the two compounds. If the pH was increased, making the phosphate more protonized, the ammonium formate would loose the ammonium ion. This means that the buffer can not be removed by freeze drying. If the pH was decreased, the GXOS would not be protonized enough to be isolated from the hydrolysate. This means that if the phosphate should be separated from GXOS, most likely, it has to be done in two steps: First, GXOS and phosphate is isolated from the wheat bran hydrolysate by anion exchange at pH 6.5, secondly the GXOS can be separated from phosphate by anion exchange by anion exchange at lower pH.

Another way to remove the phosphate, could be to precipitate it by calcium ions by addition of i.e.  $\text{Ca}(\text{OH})_2$  (Hosni et al., 2008). Calcium hydroxide has some advantages compared to other metal salts since it is rather soluble and its usage does not introduce ions such as  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Al}^{3+}$  or  $\text{Fe}^{3+}$ . Moreover, the alkalinity of the solution does not require the NaOH addition which usually is necessary for the precipitation. However, this method would require some analytical work to determine the optimal operating conditions like Ca/P molar ratio, time and pH of the reaction.

For the future work, there are several considerations concerning, how to improve the yield of the detected GXOS, once the RGI and phosphate have been removed. The arabinoxylan could be extracted from the wheat bran e.g. by the hydrogen peroxide method (Hollmann and Lindhauer, 2005) which would make the arabinoxylan more accessible for the xylanase. Another important consideration is the length of the incubation period with the xylanase. It could be hypothesized that larger xylo-oligosaccharides might contain more glucuronic acid and therefore, might have a higher affinity for the anion exchange column. Thus, a higher GXOS could be obtained. In this work, 24h incubation was used in order to obtain a high degree of hydrolysis of the wheat bran and therefore also high product yields. However, this also meant that the xylo-oligosaccharides released will be relatively short in accordance with earlier results (Rasmussen et al., 2010; Rasmussen and Meyer, 2010). If a shorter incubation period was used, longer oligosaccharides would be present in the hydrolysate.

## **Conclusion**

GXOS have been isolated from wheat bran but an even larger amount of RGI was present in the obtained samples together with phosphate. Therefore, further purification has to be made



before GXOS with a defined structure can be isolated. The finding of RGI in wheat bran is somewhat surprisingly, since only a very limited number of reports on pectic substances in wheat bran have been made.

## Chapter 7 Concluding remarks and future perspectives

The work presented in this PhD thesis has generally provided a better understanding of the enzyme kinetics and quantitative phenomena of the hydrolysis of xylan substrates by selected pure enzyme preparations primarily the BsX<sub>mut</sub> and BsX. Furthermore, the options for producing specific substituted xylo-oligosaccharides from selected substrates by specific xylanase treatment have been examined.

The enzymatic hydrolysis of WEAX by the cooperative action of BsX<sub>mut</sub>,  $\beta$ -xylosidases and two arabinofuranosidases having different specificities was examined in relation to arabinose and xylose release. The underlying hypothesis was that it was either the substitution extent or the number of non-reducing ends that was rate-limiting for the xylose release. The results demonstrated that the xylanase activity is the dominating rate-limiting enzyme activity for the xylose release. In other words, the provision of more (unsubstituted) non-reducing ends resulting from xylanase action was more efficient to boost the  $\beta$ -xylosidase activity than provision of more (randomly) unsubstituted xyloses in the arabinoxylan backbone.

The BsX<sub>mut</sub> exhibited a significant synergistic effect on xylose release when acting together with the  $\beta$ -xylosidase and comparably, the two assessed arabinofuranosidases also exhibited synergy with the  $\beta$ -xylosidase on the release of xylose. These results confirmed the hypothesis that there was correlation between the extent of substrate substitution and the enzymatic solubilization of xylan. This hypothesis was also confirmed for the BsX<sub>mut</sub> and BsX alone, when the activity on various xylan containing substrates was compared. For both enzymes the catalytic effects and rates on substrates that differ in complexity, could be related to the general structure of the active site, where substituents present a hindrance.

The exact influence of the double mutation, D11F/R122D, on the activity of BsX<sub>mut</sub> and the enzyme's selectivity as opposed to BsX, was investigated. It was established that the overall activity of BsX<sub>mut</sub> was significantly reduced on different polymeric xylan substrates, which based on the structure analysis was proposed to be due to the steric hindrance caused by the D11F mutation. This confirms the hypothesis about the double mutation having an effect on the kinetics of BsX<sub>mut</sub> as opposed to BsX. A more in depth characterization of the xylanase substrate selectivity was made, where the initial and the end point selectivity were estimated for both enzymes. The overall selectivity of BsX and BsX<sub>mut</sub> were similar indicating that the mutation did not affect the xylanases' preference for WU-AX over WE-AX. The hypothesis regarding the effect of the mutations on the selectivity was therefore rejected. Furthermore, for BsX, the addition of TAXI led to a higher degree of inhibition when incubated with WE-AX. The finding that one amino acid change at the entry to the active site,

did in fact alter the specificity, may allow for new protein enzyme design that can promote the enzyme catalyzed liberation of certain desirable product profiles in future applications.

In order to assess the heterogeneous structure of the substrate matrix and the change occurring during the xylanolytic reaction, the possibility of using HPSEC as a quantitative method was examined. The results confirmed that it was possible to exploit HPSEC profiles to obtain a quantitative, or at least semi-quantitative, insight into the evolution of the xylo-oligosaccharides during BsX treatment of xylan. Thus the underlying hypothesis was confirmed. A prerequisite for this new method was that the interference from the sample solution was reduced to an acceptable minimum which could be done by appropriate selection of the mobile phase. The xylanolytic activity could now be assessed concerning the capability to catalyze the depolymerization of the substrate and the following release of smaller and larger xylo-oligosaccharides. It is our belief that the quantitative approach reported here may be applied to other types of carbohydrate substrates and hydrolysates.

Since the arabinoxylan from flaxseed had an interesting structure (high degree of doubly substituted arabinofuranosyl), this substrate was evaluated as a potential substrate for the BsX under the hypothesis that flaxseed mucilage could be used as a substrate for the enzymatic production of xylo-oligosaccharides. The xylanase had only limited solubilising capacity of the flaxseed mucilage and was only able to depolymerise the xylan backbone to a limited extent. The limited degradation may be ascribable to the high proportion of substitution of the flaxseed xylan, compared to e.g. wheat arabinoxylan. This indicated that for future studies, enzyme mixtures containing different kinds of enzyme activities will be necessary for the solubilisation and/or depolymerization of the flaxseed arabinoxylan. Flaxseed mucilage was found to contain endogenous  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activity being active on synthetic substrates like *p*NPX and *p*NPA and natural substrates like birchwood xylan, WEAX and WUAX.

Wheat bran was also evaluated as a potential substrate for the enzymatic production of xylooligosaccharides notably GXOS. The obtained results showed that GXOS could be purified by use of anion exchange chromatography from wheat bran, but an even larger amount of RGI was present in the obtained samples together with phosphate. Therefore, further separation has to be made before GXOS with a defined structure can be isolated. The finding of RGI in wheat bran is somewhat surprisingly and therefore this is worth examining further.

When taken together, the obtained results in this PhD project have provided a better understanding of the modification and degradation of xylan containing substrates by specific

enzyme treatment. This knowledge is a prerequisite for optimally exploiting enzyme reactions in new food and ingredient processes and for exploiting biomass rationally.

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